STUDIES ON THE BIOLOGY OF ENVIRONMENTALLY PERSISTENT *LISTERIA MONOCYTOGENES* STRAINS

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STATEMENT ON THE CONTRIBUTION OF OTHERS

I acknowledge the assistance of Associate Professor Tom Ross, Dr Lyndal Mellefont and Mr Roger Latham with factory sampling, initial strain typing and provision of *Listeria monocytogenes* isolates used in this study as detailed in Chapter 2. I also acknowledge the assistance of Dr John Holah from the Chorleywood Research Centre, U.K., for provision of a *Listeria monocytogenes* isolate used in this study. Finally, I acknowledge the assistance of Dr David Ratkowsky with statistical analysis.

Rolf Erik Nilsson, December, 2010

STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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ABSTRACT

Listeria monocytogenes is a saprophytic bacterium capable of causing serious foodborne human disease. Persistent contamination by *L. monocytogenes* represents a threat to public health and has serious economic implications for the contaminated facility. Actions taken to minimise contamination of the factory environment/food products are expected to cause physiological stress to *L. monocytogenes*, and it has been shown that the ability to resist stress, and therefore persist within an environment, can augment *L. monocytogenes* virulence.

In this dissertation, the hypothesis that physiological adjustment by *L. monocytogenes* facilitates persistent food factory contamination, and that some strains are better able to implement this shift than others, is assessed. Isolates recovered from a food factory were characterised using multilocus sequence typing, and attributes of this group thought to enable persistent environmental contamination were compared. These included biofilm production and the mechanisms affording alkaline stress adaptation.

Biofilm production by *L. monocytogenes* strains isolated from multiple environments, including a food processing factory and environmentally persistent/sporadic factory isolates, due to temperature and pH stress was assessed using a colourimetric assay and scanning electron microscopy. A temperature-specific biofilm production response was observed as was environmentally induced homogeneous biofilm production by non-clonal *L. monocytogenes* strains recovered from the same environment. Observations provided evidence for distinct, inducible biofilm production. Importantly, it was concluded that biofilm production alone does not determine the persistent *L. monocytogenes* phenotype.

To investigate if stress adaptation contributes to persistent factory contamination, protein expression by alkaline adapted persistent and sporadic factory contaminants was compared using high resolution proteomics. Additionally, a well characterised *L. monocytogenes* strain was studied at pH 9.0, with emphasis on the role of cell membrane proteins. Qualitative and relative protein abundances were compared through functional ontology and determination of spectral abundances.

Findings suggested alkaline adaptation involves cytoplasmic acidification by surrogate proton sources, an energy metabolism shift, stabilisation of cellular processes and cell wall modification. This was most pronounced in exponential

phase. Although more pronounced in persistent strains, no difference in the mechanisms supporting alkaline tolerance was evident between strains.

The results of this dissertation support the notion that exposure to environmental stresses within food facilities can induce or select the persistent *L. monocytogenes* phenotype. This may be caused by the complexity of operations within these facilities, leading to concentration fluxes of cleansing/sanitising agents and other growth limiting challenges, subjecting the strains to varying forms of sublethal stress.

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LIST OF ABBREVIATIONS

a_w	Water activity
ANOVA	Analysis of variance
AlTR	Alkaline tolerance response
ATCC	American Type Culture Collection
BHI	Brain heart infusion
BURST	Based upon related sequence type
CDA	Communicable Diseases Australia
Cfu	Colony forming unit
CPAS	Computational Proteomics Analysis System
df	Degrees of freedom
EHEC	Enterohaemorrhagic *Escherichia coli*
ETC	**Electron transport chain**
FPP	Food processing plant
G_{adj}	Adjusted G - statistic
GIT	Gastrointestinal tract
JCVI – CMR	J. Craig Venter Institute Comprehensive Microbial Resource
KEGG	Kyoto Encyclopedia of Genes and Genomes
LLO	Listeriolysin O
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MS	Mass spectrometry
MudPIT	Multidimensional protein identification technology
NNDSS	National Notifiable Diseases Surveillance System
OD	Optical density
PCA	Principle component analysis
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PVC	Polyvinyl chloride
RAPD	Randomly amplified polymorphic DNA
Rep – PCR	Repetitive element polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SCX	Strong cation exchange
SEM	Scanning electron microscopy

ST	Sequence type
TE	Trisethylenediamine tetra-acetic acid buffer
t - LSD	t – test least significant differences
TPP	Trans Proteomic Pipeline
UPGMA	Unweighted pair group method with arithmetic averages
UTAS – FSC	University of Tasmania Food Safety Centre
VTEC	Verotoxigenic *Escherichia coli*

CHAPTER 1
LISTERIA MONOCYTOGENES AS A PATHOGEN AND ENVIRONMENTAL CONTAMINANT

1.1 *Listeria monocytogenes* and Public Health

Listeria monocytogenes is a ubiquitous saprophytic bacterium capable of causing serious disease in humans. The organism has been recognised for 84 years, known as a human pathogen for approximately 80 years, and a foodborne aetiology was confirmed 27 years ago (Murray *et al.,* 1926; Schlech *et al.*, 1983). Despite this, an average of 60 cases of Listeriosis are reported annually in Australia, and the incidence of this disease, along with all of the major foodborne diseases, appears to be increasing (National Notifiable Diseases Surveillance System (NNDSS); Communicable Diseases Australia (CDA); Figure 1.1).

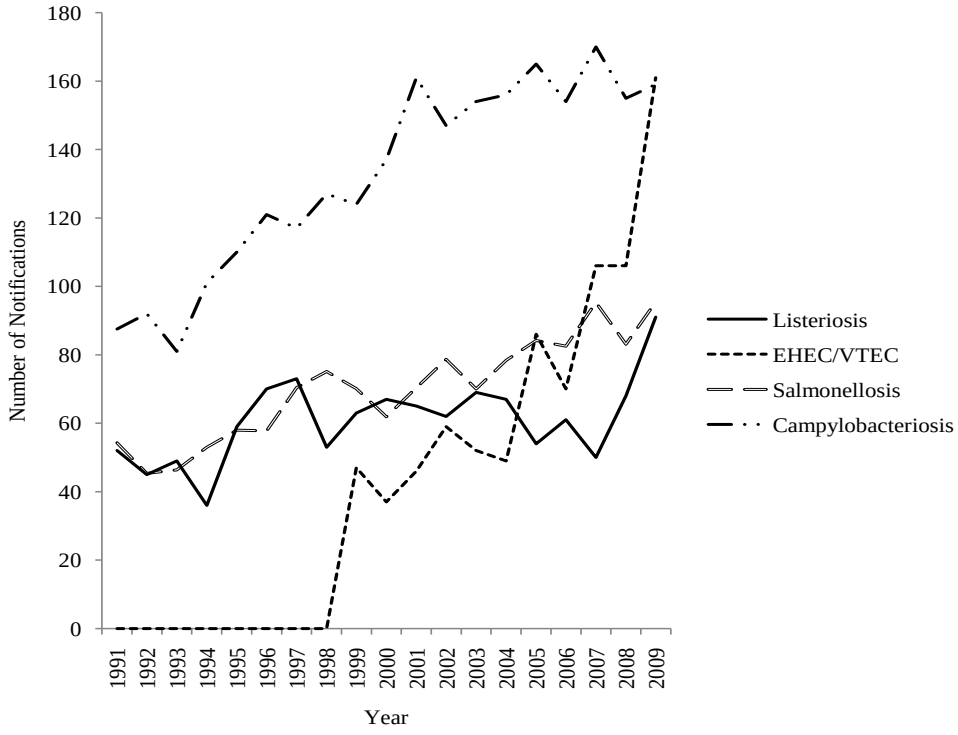


Figure 1.1 Listeriosis, Salmonellosis, Campylobacteriosis, Enterohaemorrhagic and Verotoxigenic *Escherichia coli* (EHEC / VTEC) notifications in Australia between 1991 and 2009. The number of notifications for Salmonellosis and Campylobacteriosis have been normalised for charting purposes, and are higher than the value represented on the Y - axis (100×). Notification records for EHEC / VTEC begin at 1999. Adapted from data available at the NNDSS http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-pubs-cdi-cdiintro.htm.

A substantial body of knowledge on the ecology, physiology and pathology of *L. monocytogenes* has been established, however, many gaps remain. Explanations for *L. monocytogenes'* apparent incongruent environmental distribution, for example, particularly in terms of observed hyper – contaminated agricultural settings, has not progressed far beyond the known carriage and excretion by wild, domesticated, and farm animals (Nightingale *et al.,* 2004; Andrzejewska *et al.,* 2004; Al – Ghazali and Al – Azawi, 2008). Similarly, although it is recognised that high bacterial loads are required for infection, and that healthy adults rarely acquire the invasive form of *L. monocytogenes* infection, infections acquired from low bacterial loads and invasive disease in healthy adults are reported (Chen *et al.,* 2003; Franciosa *et al.,* 2001; Gellin *et al.,* 1991; Hass *et al.,* 1999).

Despite this, *Listeria* research has resulted in many positive public health outcomes, including preventative medicine, improved food preservation strategies, and limited control within food factory environments. However, the recent increase in Listeriosis notifications in Australia (Figure 1.1), a mortality rate of up to 30%, and the economic ramifications resulting from food industry contamination and product recalls, suggest that much more research is required. In particular, the interaction between *L. monocytogenes* and the environment, and how this can manifest as virulence, environmental persistence, and increased stress resistance, is of importance to both the food industry and public health. Understanding the sources, distribution and growth limits of *L. monocytogenes* has formed a logical foundation for interventions and preventative measures directed against Listeriosis (Chasseignaux *et al.,* 2002; Crepet *et al.,* 2007; Fenlon *et al.,* 2008; Tienungoon *et al.,* 2000). Studies aimed at understanding the physical and biological aspects contributing to the distribution of both disease and the organism are areas of active *L. monocytogenes* research (Gray *et al.,* 2004; Gray *et al.,* 2006; Nightingale *et al.,* 2004; O'Toole, 2004; Toledo – Arana *et al.,* 2009). While knowledge of the ecophysiology of *L. monocytogenes* is growing, much remains to be defined. More complete knowledge on the physical and biological parameters contributing to the distribution and persistence of this organism is necessary, and could facilitate prediction of the clinical incidence of Listeriosis, limit human exposure, and provide logical strategies to prevent contamination of foods and consequent *L. monocytogenes* infections.

1.2 *Listeria monocytogenes*

1.2.1 The *Listeria* genus

The *Listeria* genus comprises motile, regular, non-sporing, non-capsulated, Gram-positive cocco-bacillus shaped bacteria (Seeliger and Jones, 1986). Size ranges from 0.4 – 0.5 µm wide and 0.5 – 2.0 µm long (Seeliger and Jones, 1986). Currently seven species are recognised including *L. monocytogenes* (the type species), *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. ivanovii* and *L. marthii* (Boerlin *et al.*, 1992; Seeliger and Jones, 1986; Graves *et al.*, 2009). *Listeria ivanovii* is further divided into two subspecies; *L. ivanovii* subspecies *ivanovii*, and *L. ivanovii* subspecies *londoniensis* (Boerlin *et al.*, 1992). Two definitive pathogens are among the *Listeria* genus. These are *L. ivanovii*, an animal pathogen, and *L. monocytogenes*, an intracellular human food-borne pathogen (Murray *et al.*, 1926; Seeliger *et al.*, 1984).

1.2.2 Subtyping of *Listeria monocytogenes*

A range of methods have lead to the identification of multiple subtypes of *L. monocytogenes*. The methods used are diverse and include a variety of immunological and DNA-based techniques (Table 1.1). While each has its merits and limitations, serotyping has been widely embraced as a general differentiation scheme for *L. monocytogenes*. To date 13 serovars have been discerned based on somatic (O) and flagellar (H) antigens (Seelinger and Hoehne, 1979; Table 1.2). All serovars have the propensity to cause disease; however a small number (serotypes 4b, 1/2a and 1/2b) have been implicated in the majority of human *L. monocytogenes* infections (Lukinmaa *et al.*, 2003).

Table 1.1 Examples of subtyping methods used to differentiate strains of *Listeria monocytogenes*.

Method	Basis	Reference
Serotyping	Differentiates strains (serotypes) based on the presence of somatic and flagellar antigens	Seelinger and Hoehne (1979)
Phage Typing	Compares the susceptibility of strains to specific bacteriophage	Rocourt *et al.* (1985)
Multilocus Enzyme Electrophoresis (MLEE)	Compares the electrophoretic mobility of the amplicon produced from PCR of multiple "house-keeping" enzymes from different strains	Boerlin and Piffaretti (1991)
Multilocus Sequence Typing (MLST)	Compares the DNA sequences of multiple "housekeeping" loci; essentially represents a DNA sequence based version of MLEE	Maiden *et al.* (1998)
Pulsed Field Gel Electrophoresis (PFGE)	Differentiates strains based on the electrophoretic pattern generated from restriction enzyme (multiple) digestion of genomic DNA, followed by subjection to multi-directional, pulsed electric fields	Brosch *et al.* (1991)
Ribotyping	Comparative analysis of ribosomal RNA gene fragments from different strains	Rocourt (1985)
Randomly Amplified Polymorphic DNA (RAPD)	Compares strains based on the DNA electrophoretic fingerprint generated from PCR amplification of polymorphic DNA regions	Czajka and Batt (1994)
Repetitive Element Polymerase Chain Reaction (Rep - PCR)	Compares the electrophoretic fingerprint based on PCR amplification using capricious primers	Jersek *et al.* (1999)
Restrictriction Fragment Length Polymorphism (RFLP)	Differentiates strains based on the polymorphism of DNA fragment lengths derived from restriction enzyme digestion of gene (often virulence related) amplicons.	Vines *et al. (*1992)
Actin A (*actA*) Analysis	Compares DNA sequence polymorphism of the *actA* amplicon between strains. Uses both direct polymorphic DNA sequence analysis and electrophoretic fingerprints.	Bania *et al.* (2009)

Table 1.2 The O and H antigens detectable on serovars of *Listeria monocytogenes* (adapted from Sutherland and Porrit, 1997).

Serovar	O - Antigens											H - Antigens			
1/2a	I	II	III[a]									A	B		
1/2b	I	II	III[a]									A	B	C	
1/2c	I	II	III[a]										B		D
3a		II	III[a]	IV								A	B		
3b		II	III[a]	IV							XII[a]	XIII[a]	A	B	C	
3c		II	III[a]	IV							XII[a]	XIII[a]		B		D
4a			III[a]		V		VII						A	B	C	
4ab			III[a]		V	VI	VII		IX	X			A	B	C	
4b			III[a]		V	VI							A	B	C	
4c			III[a]		V		VII						A	B	C	
4d			III[a]		V[a]	VI		VIII					A	B	C	
4e			III[a]		V	VI		VIII[a]	IX[a]				A	B	C	
7			III[a]								XII	XIII	A	B	C	

[a] Not present in all strains.

Based on molecular subtyping studies, *L. monocytogenes* is currently further divided into three broad evolutionary groups, termed phylogenetic lineages I, II and III (Wiedmann *et al.*, 1997; Piffaretti *et al.*, 1989). These lineage were initially determined by combinational comparison of both the ribotype pattern, and RFLP analysis of the haemolysin (*hly*), internalin A (*inlA*) and Actin A (*ActA*) genes of 133 *L. monocytogenes* strains (Wiedmann *et al.*, 1997). Since that study, a number of works have both confirmed, and further resolved, this grouping system (Bania *et al.*, 2009; Roberts *et al.*, 2006; Gray *et al.*, 2004; Zhang *et al.*, 2003).

The lineage I cluster includes *L. monocytogenes* serotypes 1/2b, 3b, 4d, 4e and most strains of serotype 4b. Serotypes 1/2a, 1/2c, 3a and 3C cluster within lineage II, while the lineage III cluster includes serotypes 4a, 4c and some serotype 4b strains (Nightingale *et al.*, 2005; Call *et al.*, 2003; Zhang *et al.*, 2003). The lineage III cluster is very rare and has been divided into three subgroups termed lineages IIIA, IIIB and IIIC based on genotypic and phenotypic characterisation (Roberts *et al.*, 2006). It is thought that the genetic, and subsequent phenotypic, characteristics differentiating the lineage III subtypes may be the result of this group occupying a unique ecological niche (Roberts *et al.*, 2006). Interestingly, a correlation between lineage and isolate source has been defined, with lineage I strains significantly more common from human clinical sources (largely due to the presence of the epidemic

serotype 4b in the lineage), lineage II strains significantly more common from food sources, and lineage III isolates rarely recovered from either food or humans (Gray *et al.*, 2004). Serotype 1/2a (lineage II) is also implicated in a large number of human listeriosis cases, second only to serotype 4b, however these are generally sporadic rather than epidemic cases (Doumith *et al.*, 2004).

To date, the genomes of six *L. monocytogenes* strains have been completely sequenced, however, genome annotation for these strains remains incomplete, with large portions remaining hypothetical and of unknown function (Table 1.3). Determination of the genome sequence of at least eighteen more strains is nearing completion at the time of writing.

Table 1.3 Strains of *L. monocytogenes* with completely sequenced genomes.

Strain	Serotype	Lineage	Genome Size (bp)	Genes		
				Protein Coding	RNA	Total
EGD-e[a]	1/2a	II	2944528	2846	81	2846
F2365[a]	4b	I	2905187	2848	85	2933
HCC23[b,c]	4a	III	2976212	2974	85	3060
Clip80459[b,c]	4b	I	2912690	2766	85	2973
08-5578[b]	1/2a	II	3109342	3088	73	3162
08-5923[b]	1/2a	II	2999054	2966	73	3039

a Glaser *et al.* (2003)
b KEGG Genome
c National Centre for Bioinformatics

L. monocytogenes strains display considerable genetic diversity (Call *et al.*, 2003). Genome diversification is thought to be important to the species, particularly in regards to genes encoding proteins associated with the cell surface (Bierne and Cossart, 2007; Zhang *et al.*, 2003). Cell surface proteins are abundant in *L. monocytogenes*, (133 identified in *L. monocytogenes* strain EGD-e) and the diversity they exhibit is thought to reflect the organism's ability to survive in a range of environments, including hosts during infection (Bierne and Cossart, 2007). Genetic diversification resulting from occupation of specific microenvironments is a pattern characteristic of many environmental microbes (Lorenz and Wackernagel, 1994; McArthur *et al.*, 1988). Importantly, ≈ 23% of the genes encoding surface proteins in *L. monocytogenes* strain EGD-e are absent from the genome of the non – pathogenic *L. innocua* strain CLIP11262, in contrast with only ≈ 10% of the total

L. monocytogenes strain EGD-e genes being absent from *L. innocua* strain CLIP11262 (Glaser *et al.*, 2001).

1.3 Infection and Disease

1.3.1 Reservoir, host range, acquisition and transmission

L. monocytogenes is a ubiquitous saprophyte and is known to be associated with soil and decaying plant matter (Fenlon, 1999; Botzler *et al.*, 1974; Weis, 1975). This association is believed to contribute to the inclusion of many animals (including ruminants, birds, insects, fish, crustacean and humans) in an ecological cycle in which the organism is consumed during feeding on contaminated soils and vegetation, carried to new sites and excreted, subsequently dispersing the organism into new environments (Nightingale *et al.*, 2004; Sutherland and Porritt, 1997; Figure 1.2). The prevalence of *L. monocytogenes* within animals differs, however, depending on the environment in which the animal has grown, and a number of other factors, including the stage of growth and diet (Nightingale *et al.*, 2004; Fenlon *et al.*, 2008; Wesley *et al.*, 2008). Animals, particularly those reared for meats and other products destined for human consumption, represent a primary source for *L. monocytogenes* transmission to humans (Nightingale *et al.*, 2004; Tauxe, 1997).

L. monocytogenes has long been recognised as a human pathogen (Murray *et al.*, 1926). Infection was initially thought to originate from direct contact with soil or animals contaminated by *L. monocytogenes*. Although this has been demonstrated, recognition of *L. monocytogenes* as a human food-borne pathogen occurred in the early 1980's, and is now accepted as the primary means of infection based on the large body of evidence produced since that time (e.g. Schlech *et al.*, 1983; Nightingale *et al.*, 2004; Czajka and Batt, 1994; Fenlon *et al.*, 2008; Maklona *et al.*, 2010).

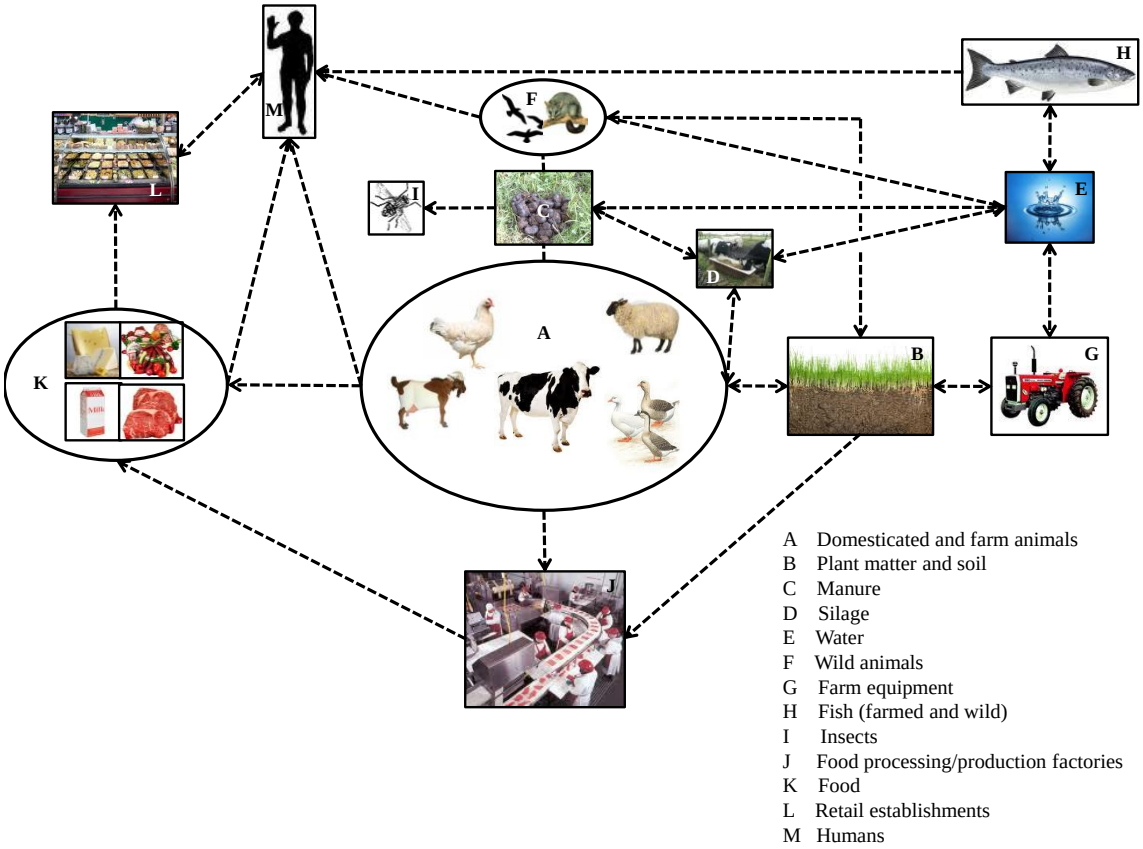


Figure 1.2 A descriptive model outlining the ecological (and transmission) cycle of *L. monocytogenes.*

1.3.2 Infection and disease manifestation

Infection by *L. monocytogenes* can result in invasive or non-invasive disease (Franciosa *et al.*, 2001; Schlech, 1997). Non-invasive infection is the most common clinical manifestation. It is often asymptomatic or presents as a self limiting "influenza-like" infection characterised by fever, diarrhea, headache, muscle and joint pain, and emesis (Ooi - Say and Lorber, 2005). The invasive form, often termed listeriosis, is a potentially fatal dissemination characterised by sequelae such as meningitis, septicaemia and spontaneous abortion in pregnant females. This form of the disease is rare, with one study reporting fewer than ten cases per million people, with an average of 60 cases a year reported in Australia; however the mortality rate of listeriosis can range from 14 - 30% (Denny *et al.*, 2007; Gellin *et al.*, 1991; NNDSS, CDA, 2010).

In healthy adults, infection generally requires ingestion of a large inoculum and results in non-invasive disease (Chen *et al.*, 2003; Franciosa *et al.*, 2001; Ooi - Say and Lorber, 2005; Food and Agriculture Organisation/World Health

Organisation of the United Nations, 2004). However, in pregnant women, the very old, the very young, immunocompromised individuals infection may be acquired more easily and can result in invasive disease (Vázquez-Boland *et al.*, 2001; Franciosa *et al.*, 2001; Ooi - Say and Lorber, 2005; Schlech, 1997; Food Agricultural Organisation / World Health Organisation of the United Nations 2004). Infection by *L. monocytogenes* is believed to occur primarily within the gastrointestinal tract (G.I.T.) (Kim *et al.*, 2004; Karunasagar *et al.*, 1994). The clinical symptoms of non – invasive illness generally present after ≈20 hours, while symptoms associated with the invasive form may not present for up to thirty days (Vazquez – Boland *et al.*, 2001). It is believed that a very high dose ($\approx 10^6$ cells / g) of *L. monocytogenes* is generally required for infection; however doses as low as 10^2 cells / g have been associated with invasive infection, and strain variation in infective potential is reported (Chan and Wiedmann, 2009; Vázquez-Boland *et al.*, 2001; Lukinmaa *et al.*, 2003; Schmidt – Hempel and Frank, 2007; Haas *et al.*, 1999).

Knowledge of the mechanism of invasive infection by *L. monocytogenes* is increasing. Briefly, *L. monocytogenes* invades the intestinal epithelium and Peyers patches following passage through the stomach (Sleator *et al.*, 2009; Vázquez-Boland *et al.*, 2001; Karunasagar *et al.*, 1994). Endothelial cell invasion follows, facilitated by the internalin proteins, or by cell – to – cell spread after uptake by macrophage (Sleator *et al.*, 2009; Vázquez-Boland *et al.*, 2001). Once past the intestinal barrier, *L. monocytogenes* disseminates via the lymph / blood system to lymph nodes, the spleen, or the liver (Vázquez-Boland *et al.*, 2001). Macrophages in the spleen and liver (Kupffer cells) rapidly clear *L. monocytogenes* from the blood stream with 90% of the bacteria being trapped in the liver by Kupffer cells (Vázquez-Boland *et al.*, 2001).

Kupffer cells are part of the innate immune response to *L. monocytogenes* which inhibits replication during the first 2-3 days of infection, and are responsible for induction of an acquired immune response (Portnoy *et al.*, 2002). Neutrophils, attracted by chemokines secreted by the Kupffer cells, adhere to the Kupffer cells and destroy both extracellular *L. monocytogenes* and infected host cells (Conlan, 1997). Most viable *L. monocytogenes* have been internalised by hepatocytes within six hours, and this appears to be the preferred site of intracellular replication (Vázquez-Boland *et al.*, 2001).

Hepatocytes actively destroy both the internalised *L. monocytogenes* cells and themselves (autophagy) in an attempt to eliminate infection (Sleator *et al.*, 2009).

The reasons why some *L. monocytogenes* cells avoid autophagy has, until recently, been unknown. In a recent study it was shown that *L. monocytogenes* can use Actin A (ActA) to disguise itself from the host cell by recruiting host cell proteins and, subsequently, avoiding recognition and preventing autophagy (Yoshikawa *et al.*, 2009). Those *L. monocytogenes* cells that are able to survive rapidly proliferate until checked by acquired immunity (Portnoy *et al.*, 2002; Gahan and Hill, 2005). The cells that survive may be better able to undergo the physiological shifts (e.g. adjustment of metabolic systems, alteration of the cell wall, increased expression of cell surface structures) necessary for adaptation to stressful environments, and may explain some of the observed variation and functional overlap in stress tolerance and virulence in *L. monocytogenes*, including the presence or absence of virulence factors between strains (Toledo-Arana *et al.*, 2009; Gray *et al.*, 2006; Gray *et al.*, 2004).

L. monocytogenes cells then spread cell – to – cell via actin-based motility, avoiding exposure to the host's humoral immune response (Portnoy *et al.*, 2002). After approximately four days the infection is confined by granuloma, however shedding may occur allowing dissemination via the blood stream to other sites within the host, including the uterus (and unborn foetus), central nervous system, brain and other organs (Portnoy *et al.*, 2002). Complete resolution of *L. monocytogenes* infection requires activation of a CD4 / CD8 T – cell response, however, infection often becomes systemic before this may be effected, resulting in the high mortality rate observed in listeriosis (Sleator *et al.*, 2009; Hiromatsu *et al.*, 1992).

1.4 Contamination by *Listeria monocytogenes*

1.4.1 Growth limits

Conditions limiting the growth of *L. monocytogenes* have been determined in many instances. These are effectively applied as controls in food production and processing settings, and as a means of preserving food products. Examples include fermented meats such as salami, which are effectively preserved by reduced pH and decreased water activity; canning, preserved by heat and removal of air; and packaging in a modified atmosphere, preserved by replacing oxygen with gases such as CO_2 and nitrogen. However, a number of studies have determined that many of the conditions that stress, and subsequently limit, growth in *L. monocytogenes* can also increase virulence (Anderson *et al.*,, 2007; Bigot *et al.*, 2006; Borezee *et al.*,

2000; Gray *et al.*, 2006; Kazmierczak *et al.*, 2003). This is particularly concerning in regards to reports of induction of cross protection from one stress to another (Lou and Yousef, 1997; Lunden *et al.*, 2003; Giotis *et al.*, 2008b). Should any failings in the implementation of controls occur, many of the methods applied may actually increase the organism's propensity to cause disease.

1.4.1.1 Temperature

L. monocytogenes is capable of survival and growth at temperatures ranging from -0.4° C to 45° C (Chan and Wiedmann, 2009; Gray *et al.*, 1948; Walker *et al.*, 1990). The organisms ability to grow at refrigeration temperatures (0 – 4° C) is problematic to the food industry, with prolonged survival and growth at 4° C recorded (Chan and Wiedmann, 2009; Wilkins *et al.*, 1972). Optimal growth by *L. monocytogenes* occurs between 30° C and 37° C (Cole *et al.*, 1990; Wilkins *et al.*, 1972). Importantly, studies have shown that growth at optimal and elevated temperatures can impart a protective effect against subsequent exposure to environmental stresses such as pH, salt and disinfection (Kastberg *et al.*, 2009; Kagkli *et al.*, 2009; Cole *et al.*, 1990; Patchet *et al.*, 1996).

As with all inactivation processes, temperature inactivation is stochastic, with the rate of inactivation increasing by 10% (approximately) for every 6° C increase in temperature (Mackey and Bratchell, 1989). Further to this, the inactivation rate depends on the size of the initial inoculum, as well as other properties of the growth matrix (Mackey and Bratchell, 1989). With an inoculum of approximately 10 colony forming units (cfu)/g, studies have shown that temperatures between 67 and 69° C will reduce the population by 90% in a few seconds, and eliminate realistic contamination levels completely in 30 – 60 seconds (Gaze *et al.*,1989; Farber, 1989; Mackey and Bratchell, 1989).

1.4.1.2 Atmosphere

L. monocytogenes is a facultative anaerobe capable of growth in anaerobic, microaerobic and aerobic environments (Lungu *et al.*, 2009). Although predominantly aerobic, under certain conditions the organism favours anaerobic metabolism, particularly during recovery from sublethal damage and stress (Lungu *et al.*, 2009; Buchanan and Klawitter, 1990). This offers a selective advantage in

environments prone to atmospheric shifts and is reported to aid intracellular survival and immune system evasion, thus contributing to virulence (King *et al.,* 2003; Anderson *et al.,* 2007). *L. monocytogenes* can grow, at a slower rate and with increased lag times, in modified atmospheres including up to 40% CO_2, with good growth in atmospheres containing up to 75% CO_2 reported when small amounts of oxygen are also present (Anderson *et al.,* 2007; Lungu *et al.,* 2009). This has serious implications for food industry, especially those that use modified packaging systems. Particularly concerning are reports that the ability of *L. monocytogenes* to invade cells increases in low oxygen environments (Anderson *et al.,* 2007). Consequently, atmospheric conditions can dictate not just growth and survival, but also the propensity to infect and therefore cause disease.

1.4.1.3 Hydrostatic Pressure

Hydrostatic pressure, both thermal and non – thermal, is applied as a control against *L. monocytogenes* as it can inactivate the organism while minimising deterioration of food quality attributes (Toepfl *et al.,* 2006; Chen *et al.,* 2009). While the limits of barotolerance in *L. monocytogenes* remain to be fully defined, it is understood that barotolerance differs between strains of *L. monocytogenes*, and pressures of ≥600 Mpa have been recommended for control (Tay *et al.,* 2003; Chen *et al.,* 2009). Cross protection to hydrostatic pressure has been reported, with stationary growth phase and the nature of the growth medium (particularly media of high osmotic pressure) suggested to increase survival (Bowman *et al.,* 2008; Styles *et al.,* 1991; Simpson and Gilmour, 1997).

1.4.1.4 Water activity

Water activity (a_w) is a measure of the osmotic potential of a growth medium; defined as the ratio of vapour pressure of a growth medium (at equilibrium with the surrounding atmosphere) to the vapour pressure of pure water under the same conditions (Sutherland and Porritt, 1997). Essentially, a_w represents the water available for growth. *L. monocytogenes* has a minimum water activity requirement of between 0.91 and 0.93, depending on the solute controlling a_w, and the *L. monocytogenes* strain (Farber *et al.,* 1992; Ross *et al.,* 2000). Optimal a_w for the growth of *L. monocytogenes* has been determined to be between 0.98 and 0.99

(Tienungoon *et al.,* 2000). As with other growth limiting controls, tolerance of decreased water activity has been shown to increase following exposure to other stresses, including pH and NaCl stress (Giotis *et al.,* 2008; Vogel *et al.,* 2010). Importantly, tolerance of decreased a_w is now thought to be an important factor contributing to environmental persistence by *L. monocytogenes* strains (Vogel *et al.,* 2010).

1.4.1.5 Nutrition

The specific nutritional requirements of *L. monocytogenes* remain poorly understood. However, a synthetic minimal growth medium was recently developed by Tsai and Hodgson (2003). This medium was capable of supporting the growth of a range of *L. monocytogenes* strains in both liquid and solid culture, superseding previous minimal growth media (Friedman and Roessler, 1961; Premaratne *et al.,* 1991; Jones *et al.,* 1995), and defining a number of the minimal nutritional requirements for growth under laboratory, and presumably environmental, conditions. Key nutritional findings by Tsai and Hodgson (2003) included *L. monocytogenes* requirement for exogenous methionine, cysteine, riboflavin and lipoic acid. Additionally, they determined that *L. monocytogenes* could use inorganic nitrogen other than nitrate as a nitrogen source, contradicting earlier work (Premaratne *et al.,* 1991), and they established that thiamin and biotin were needed to form normal colonies on agar. Finally, it was observed that carbohydrate catabolism in *L. monocytogenes* is limited to glucose, glycerol, fructose and mannose, and that the addition of ferric citrate (0.2 μg/mL) and hemin (0.2 μg/mL) accelerated the growth rate and decreased the lag period, while addition of hemin at 20μg/mL increased the lag period with no effect on growth rate.

Tsai and Hodgson (2003) found that *L. monocytogenes* is sensitive to extreme nutrient starvation stress and high phosphate levels. This observation is (indirectly) supported by earlier work looking at the starvation survival response in *L. monocytogenes* (Herbert and Foster, 2001). Importantly, host nutrition has been shown to effect colonisation and translocation by *L. monocytogenes* within the intestine (Ebersbach *et al.,* 2010). In that study, it was observed that host consumption of xylooligosaccharides and galactooligosaccharides improved host resistance to *L. monocytogenes,* while pectin and inulin reduced it. Given the specific nutritional requirements of *L. monocytogenes* and the role host nutrition can have in

preventing infection, further work detailing the nutritional requirements of *L. monocytogenes* is required.

1.4.1.6 pH, cleansing and santising agents

A range of factors influence the pH growth limits of *L. monocytogenes*, including cross protection from other stresses, temperature, inoculum size and properties of the growth matrix (Cole *et al.,* 1990; McClure *et al.,* 1989; Tienungoon *et al.,* 2000; Vermeulin *et al.,* 2009). In general, *L. monocytogenes* has a growth permissive pH range of 4.1 – 9.6 (Cole *et al.,* 1990; Giotis *et al.,* 2008). Further to this, *L. monocytogenes* is capable of surviving pH extremes well beyond 4.1 – 9.6, particularly if preconditioned at milder pH, and if afforded cross protection from other physiological stresses such as those applied within food processing / production environments (Vasseur *et al.,* 1999; Tienungoon *et al.,* 2000; Giotis *et al.,* 2008a; Giotis *et al.,* 2008b). These include combinations of acid and alkaline cleansers and sanitisers such as tri – sodium phosphates, ammonium salts of phosphate, hydro – acetic and hydrochloric acids. Development of resistance to these regimes is largely based on the complexity of these environments leading to diminished cleansing and sanitisation agent exposure, and the protection afforded by the development of biofilms (Somers and Wong, 2004; Mafu *et al.,* 1990; Lunden *et al.,* 2002; Holah *et al.,* 2004; Thevenot *et al.,* 2005; Kastbjerg *et al.,* 2009; Pan *et al.,* 2006).

As with many other physiological stresses, adaptive and cross-adaptive resistance to, and from, cleansing and sanitising agents has been reported (Lunden *et al.,* 2003). On this basis, the rotation of cleansing and disinfection agents, routinely performed in food production and processing facilities, has been discouraged (Lunden *et al.,* 2003).

1.4.2 Contamination of foods

Foods commonly contaminated by *L. monocytogenes* include dairy products (particularly soft cheeses), meats (particularly ready to eat meats), fish (particularly gravalax and smoked fish), prepared salads, fresh fruit and vegetables (Rocourt, 1996; Alessandria *et al.,* 2010; Fenlon *et al.,* 2008; Maklon *et al.,* 2010; Crepet *et al.,* 2007).

The prevalence of *L. monocytogenes* in fresh vegetables is similar to that of ready-to-eat meats (Beuchat, 1996; Crepet *et al.,* 2007). In an extensive study

estimating the extent of contamination by *L. monocytogenes* on fresh vegetables, from 165 prevalence studies, comprising a total of 25078 samples, 3% were positive for *L. monocytogenes* (Crepet *et al.,* 2007). Similar results can be seen with fresh fruit (Heaton and Jones, 2008; Alexandra and Sofos, 2007). The contamination of meats, poultry, fish, and dairy products by *L. monocytogenes* is common, with a reported prevalence of up to 69, 62, 50 and 20% respectively (Buncic, 1991; Miettinen *et al.,* 2001; Johansson *et al.,* 1999; Waak *et al.,* 2002). Ready – to – eat versions of these products, in particular, have a high incidence of *L. monocytogenes* contamination, which is a cause for concern given that these items are likely to be consumed with no further preparation (Johnson *et al.,* 1990; Levine *et al.,* 2001; Hudson *et al.,* 1992; Rudolf and Scherer, 2001).

Contamination of the raw materials used to produce these foods fits with the proposed ecological cycle of *L. monocytogenes,* detailed in Figure 1.2, particularly in regards to the farm environment and subsequent transmission to humans. While many processes, including heat, pH, modified atmosphere and water activity, can greatly reduce the risk of *L. monocytogenes* contamination, *L. monocytogenes* is capable of surviving many of the physiological challenges imposed as controls (Maklona *et al.,* 2010; Lou and Yousef, 1997; Section 1.4.1).

Contamination of food by *L. monocytogenes* can occur at any stage of the food production and processing cycle. *L. monocytogenes* may be introduced directly from the farm or other environment, or on the raw products used to prepare processed foods (Nightingale *et al.,* 2004). Additionally, it can be present on food handling equipment such as racks and rollers, as well as pallets, forklifts, or doors and benches in processing plants (Eklund *et al.,* 1995; Lunden *et al.,* 2002). Food may be contaminated by direct contact with these surfaces, or indirectly, by contact with a person or other piece of equipment that has come into contact with the contaminated surface (Lunden *et al.,* 2002; Lunden *et al.,* 2000; Tompkin, 2002).

The risk from *L. monocytogenes* contamination in foods such as ice cream and fermented meat products that do not permit growth during appropriate storage (e.g., frozen at - 18°C) is very low (Chen *et al.,* 2003). However, in growth-permissive foods, such as milk, fresh and vacuum packaged products, the consequences of contamination are increased, particularly if subjected to prolonged storage times and transport / storage temperature abuse (Farber *et al.,* 1998; Hudson and Mott, 1993). This is of particular concern if post-processing contamination has occurred, as *L. monocytogenes* numbers may increase to a hazardous level, and the

organism can grow at refrigeration temperatures (Cole *et al.,* 1990). Furthermore, given the likelihood that the cells have been exposed to physiological stress, and the correlation between the stress and virulence responses of *L. monocytogenes*, the contaminated product may contain cells with increased virulence potential (Anderson *et al.,* 2007; Johansson *et al.,* 2002; Kazmierczak *et al.,* 2003; Lungu *et al.,* 2009).

1.4.3 Environmental contamination

Environmental contamination by *L. monocytogenes* is widely reported (Fenlon, 1999; Gray *et al.,* 2006; O'Toole, 2004; Toledo *et al.,* 2009; Watkins and Sleath, 2008). Natural contamination of soil and plant matter forms a part of the organism's ecological cycle (Nightingale *et al.,* 2004; Fenlon, 1999; Watkins and Sleath, 2009; Figure 1.2). However, studies have shown that agricultural environments can display "hyper – contamination", and that variation in prevalence of *L. monocytogenes* exists, even between similar agricultural settings and animals (Estaban *et al.,* 2009; Wesley *et al.,* 2008; Durso *et al.,* 2010). Reports have attributed this variation to a number of factors, including farm animal husbandry and diets (e.g. silage) (Hellströ *et al.,* 2010; Ho *et al.,* 2007; Nightingale *et al.,* 2004; Watkins and Sleath, 2009; Wesley *et al.,* 2008), the use of animal manure as fertiliser (Al – Ghazali and Al – Azawi, 2008; Nightingale *et al.,* 2004; Pell, 1997; Gale, 2005), the presence of wild animals (particularly birds) (Andrzejewska *et al.,* 2004; Fenlon, 1985), dissemination by farm equipment (Fenlon *et al.,* 2008; Ho *et al.,* 2007; Nightingale *et al.,* 2004; Waak *et al.,* 2002), and transmission by asymptomatic animal and human carriers (Nightingale *et al.,* 2004; Zundel and Bernard, 2006).

Contamination of farm environments provides a direct entry point into the human food supply chain (Nightingale *et al.,* 2004). Carriage by food animals, their products and associated equipment can facilitate entry, with subsequent contamination, to food processing environments (Hellstro *et al.,* 2010; Zundel and Bernard, 2006). This, in turn, provides an entry point for dissemination into human foods. Controls are available to reduce the risk of carriage of *L. monocytogenes* into the food processing environment by equipment and primary ingredients (Lado and Yousef, 2007; Silva *et al.,* 2003). However, the ability of this organism to survive many of the physiological challenges imposed as controls, as well as failings in applying the controls, can allow it to contaminate the factory environment, and subsequently, increase the risk of food contamination (Chan and Wiedmann, 2009;

Cole *et al.*, 1990; Fenlon *et al.*, 2008; Sila *et al.*, 2003). The risk inherent in food contamination is further increased if the factory produces minimally processed ready-to-eat end – products (Hill *et al.*, 2002). Examples include meat, poultry, fish and dairy processing plants, and facilities producing prepackaged salads and similar products (Alexandra and Sofos, 2007; Chasseignaux *et al.*, 2002; Chasseignaux *et al.*, 2001; Hoffman *et al.*, 2003; Silva *et al.*, 2003).

Importantly, studies have identified a distinction between the initial source of factory contamination, and contamination of the end-product by *L. monocytogenes* (Fenlon *et al.*, 2008; Hoffman *et al.*, 2003; Norton *et al.*, 2001). Raw materials entering the factory are believed to represent the primary source of factory contamination (Fenlon *et al.*, 2008, Hoffman *et al.*, 2003). However, contamination of the end product is believed to predominantly occur from the factory environment itself; suggesting a sequence of events occurs including introduction of the contaminant, colonisation of the factory environment, and finally, product contamination from sites of colonisation (Alessandria *et al.*, 2010; Hellström *et al.*, 2010; Hoffman *et al.*, 2003; Norton *et al.*, 2001).

1.4.4 Persistent factory contamination by *Listeria monocytogenes*

Persistent contamination of food production and processing plants by *L. monocytogenes* has been reported in almost all food industries, and for periods of up to 8 years (Keto – Timonen *et al.*, 2007; Chasseignaux *et al.*, 2001; Holah *et al.*, 2004; Nucera *et al.*, 2010). Persistent contamination by *L. monocytogenes* has been associated with an elevated risk of further dissemination throughout the food production / processing facility, and increased risk of systemic factory contamination and contamination of the foods produced (Holah *et al.*, 2004; Fenlon *et al.*, 2008; Hoffman *et al.*, 2003; Norton *et al.*, 2001). The persistent contaminants constitute an internal source for further factory and food contamination, independent of *L. monocytogenes* entering the facility from the external environment (Holah *et al.*, 2004). Subsequently, persistent contamination increases the likelihood of transmitting *L. monocytogenes* to humans (Alexandra and Sofos, 2007; Chasseignaux *et al.*, 2001). Multiple studies have used molecular subtyping methods (examples presented in Table 1.1) to discern persistent or "resident" *L. monocytogenes* populations within food factory environments, and persistent subtypes have been recovered from human listeriosis cases (Chasseignaux *et al.*, 2001; Fenlon *et al.*,

2008; Ho *et al.,* 2007; Holah *et al.,* 2004; Keto – Timonen *et al.,* 2007; Alessandria *et al.,* 2010; Olsen *et al.,* 2004).

L. monocytogenes strains vary in their ability to persist within factory environments (Keto-Timonen *et al.,* 2007; Lunden *et al.,* 2000; Lunden *et al.,* 2003; Rorvik, *et al.,* 1995; Fenlon *et al.,* 2008; Holah *et al.,* 2004). The physiological mechanisms contributing to environmental persistence by *L. monocytogenes* strains appear to be multi – faceted; however differential resistance to environmental stresses and the ability to produce biofilms are increasingly implicated as characteristics favouring persistence (Abram *et al.,* 2008a; Begley *et al.,* 2009; Borucki *et al.,* 2003; Chae and Schraft, 2000; Chan and Wiedmann, 2009; Gahan *et al.,* 1996; Harvey *et al.,* 2007; Hill *et al.,* 2002; Kagkli *et al.,* 2009; Keto-Timonen *et al.,* 2007). *L. monocytogenes* has a robust physiology capable of supporting viability and growth under a range of adverse conditions (Section 1.4.1) and exposure to adverse conditions at levels that permit survival can impart a protective effect against subsequent exposure to that, and other, physiological stresses (Lou and Yousef, 1997; Lunden *et al.,* 2003; Giotis *et al.,* 2008b).

Multiple studies and reviews have investigated the stress responses of *L. monocytogenes* (Cole *et al.,* 1990; Gandhi and Chikindas, 2007; Giotis *et al.,* 2007a; Giotis *et al.,* 2007b; Giotis *et al.,* 2008; Hill *et al.,* 2002; Kazmierczak *et al.,* 2003; Kastberg *et al.,* 2009; Lou and Yousef, 1997; Patchet *et al.,* 1996). To date, those studies have been unable to confidently identify physiological patterns characteristic of environmentally persistent subpopulations of *L. monocytogenes.* Some *L. monocytogenes* strains are better able to resist environmental stresses than others, but to what extent this manifests as an increased ability to contaminate a food factory for extended time periods remains to be determined (Kagkli *et al.,* 2009; Keto – Timonen *et al.,* 2007; O'Driscoll *et al.,* 1996; Tay *et al.,* 2003).

The development of resistant subpopulations *within* a single population of *L. monocytogenes* cells could lead to diversity of stress resistances in *L. monocytogenes.* This was recently assessed by Kastberg *et al.* (2009) who tested a population of clonal *L. monocytogenes* cells to see if some members developed resistance to disinfection stress. This was based on the routine use of these agents within these food processing facilities, and a commercial, acidic, disinfectant was used in the study. If some members of a single population were better able to resist the disinfectant regime employed within a given facility, this population may be selected for, and could establish itself as a persistent contaminant. However, the

results of Kastberg *et al.* (2009) showed an homogeneous response by the population, both in terms of sensitivity and resistance, as well as the cross-protective responses induced by preconditioning the cells to alternate stresses. Similar results have been observed for other environmental challenges in both single and multi – strain studies (Lou and Yousef, 1997; Gahan *et al.*, 1996; Giotis *et al.*, 2007a), and suggest that other mechanisms, beyond resistance to environmental stress alone, permit some *L. monocytogenes* strains to persistent within a given environment, while excluding others.

The ability to form biofilm is a demonstrated means of protection against environmental stresses (Davey and O'Toole, 2000; Pan *et al.*, 2006; Donlan, 2002; Møretrø and Langsrud, 2004). A biofilm is a structured community of microbes embedded within an organic polymeric matrix that is irreversibly adhered to a surface (Davey and O'Toole, 2000). Biofilm formation by *L. monocytogenes* is well documented, and variable production by different strains is described (Borucki *et al.*, 2003; Chae and Schraft, 2000). Biofilms form a protective microbial environment while permitting essential transfer processes to occur with the outside environment. Importantly, biofilm production by *L. monocytogenes* in food production and processing environments has been demonstrated and has been shown to protect *L. monocytogenes* from cleansing and sanitising agents, desiccation, starvation and other growth limiting conditions (Pan *et al.*, 2006; Møretrø and Langsrud, 2004; Holah *et al.*, 2004). This resistant population can disseminate and further contaminate the factory as cells are released or "sloughed" from individual biofilm communities, potentially going on to attach and colonise other parts of the factory (Møretrø and Langsrud, 2004; Costerton *et al.*, 1995). It is thought that variable biofilm production by *L. monocytogenes* strains represents a key factor contributing to persistent contamination of food environments (Borucki *et al.*, 2003; Chae and Schraft, 2000; Møretrø and Langsrud, 2004).

While production of biofilm certainly contributes to survival in, and persistent contamination of, food environments, factors other than biofilm formation alone appear to permit enhanced stress resistance and persistent contamination of food production/processing environments by *L. monocytogenes* strains. Further work is necessary to identify the individual strain specific and environmental factors contributing to environmental persistence by this organism, and to determine the combination of elements that manifest as an enhanced ability to persist within food production/processing environments.

1.5 Hypothesis

The hypothesis underlying this study is that physiological adjustment (e.g. adjustment of metabolic systems, alteration of the cell wall, increased expression of cell surface structures) by *L. monocytogenes* facilitates persistent food factory contamination, and that some *L. monocytogenes* strains are better able to implement this physiological shift than others.

1.6 Proposed Research

This work aims to define biological mechanisms contributing to persistent contamination by *L. monocytogenes* of food factory environments, and seeks to delineate the persistent *L. monocytogenes* phenotype. Specifically, this work will aim to:

i) Characterise persistent *L. monocytogenes* subtypes recovered from a food factory survey using Multilocus Sequence Typing (MLST).
ii) Determine the relatedness of *L. monocytogenes* subtypes recovered from a food factory survey, and its potential to explain the extent and source of the factory contamination.
iii) Compare biofilm formation by a variety of *L. monocytogenes* strains, including persistent and non-persistent food factory contaminants, under temperature and pH stress conditions.
iv) Compare the protein expression profile of a persistent and non-persistent *L. monocytogenes* factory contaminant preconditioned to growth under alkaline conditions similar to those found in the factory from which they were recovered.
v) Characterise the physiological mechanisms underpinning the alkaline adaptation response in *L. monocytogenes* by protein expression profiling.
vi) Define the persistent *L. monocytogenes* phenotype recovered from the factory survey, based on information derived from the experiments performed in this work, and compare it with previously described persistent *L. monocytogenes* phenotypes.

CHAPTER 2
MULTILOCUS SEQUENCE TYPING OF *LISTERIA MONOCYTOGENES* RECOVERED AS PERSISTENT AND SPORADIC CONTAMINANTS OF A FOOD PROCESSING FACILITY

2.1 Introduction

Listeria monocytogenes has been implicated in many food-borne outbreaks and epidemic strains have been definitively identified (Nightingale *et al.,* 2005; Roberts *et al.,* 2009). Furthermore, persistent factory contamination over extended periods by specific strains of *L. monocytogenes*, including high risk or epidemic strains has been reported (Lunden *et al.,* 2003; Lunden *et al.,* 2002; Holah *et al.,* 2004).

Eradication of *L. monocytogenes* from foods and food processing environments remains problematic. Different strains of *L. monocytogenes* can vary in their ability to persist within a range of environments (Borucki *et al.,* 2003; Pan *et al.,* 2006; Holah *et al.,* 2004; Kagkli *et al.,* 2009). The underlying mechanisms of persistence are multi-faceted, with strain specific adaptation to physiological stresses and differential ability to form biofilm suggested (Pan *et al.,* 2006; Holah *et al.,* 2004; Chae and Schraft, 2000). Identification of persistent *L. monocytogenes* strains and knowledge of contributing parameters is fundamental for development of effective risk management, prevention and control strategies.

Clusters of *L. monocytogenes* molecular subtypes implicated in food-borne outbreaks have been reported and subtyping has proved useful in epidemiological investigation (Border *et al.,* 1990; Gray *et al.,* 2004; Jersek *et al.,* 1999; Lukinmaa *et al.,* 2003). However, "persistent" molecular subtypes remain to be definitively characterised. Molecular definition of persistent subtypes and an understanding of the underlying physiological response could identify the distribution and prevalence of high risk *L. monocytogenes* populations, and help guide preventative measures.

Molecular typing has been employed in many studies on *L. monocytogenes* (Border *et al.,* 1990; Gray *et al.,* 2004; Jeresek *et al.,* 1999; Lukinmaa *et al.,* 2003). Molecular typing methods have the power to provide answers to short term, local questions, such as the differentiation of clonal groups of pathogens; as well as long term, global questions such as determination of population ancestry. Regardless of

the method employed, a fundamental objective of molecular typing is to obtain the highest level of discrimination possible.

A succession of methods has been developed in efforts to maximise resolution and increase discriminatory power. Pulsed-field gel electrophoresis (PFGE), ribotyping and repetitive element polymerase chain reaction (rep-PCR) utilise restriction enzymes, specific and capricious primers respectively. The latter two methods measure maximal genomic variation within a given population, and any observed variation within a test population is likely to have evolved recently (Maiden *et al.*, 1998). In contrast, methods such as multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) analyse a small number of alleles on multiple housekeeping loci (Maiden *et al.*, 1998). This achieves high resolution by measuring slowly evolving variation within a test population, and allows less ambiguous characterisation of test organisms (Aanensen and Spratt, 2005). The discriminatory power of MLST and PFGE has been compared and both techniques shown to produce similar resolution (Godoy *et al.*, 2003). However, the relative ease of MLST compared to PFGE has lead to increased application; but this is off-set by the high cost involved with MLST analysis (Dyet *et al.*, 2004).

MLST has rapidly become the method of choice for many genetic relatedness studies, and is the "gold standard" method for determining the genetic relatedness of meningococci (Dyet *et al.*, 2004). The primary advantages of MLST are portability and ease of data manipulation (Aanensen and Spratt, 2005). As the technique creates nucleotide sequence data, results are easily moved between laboratories electronically. Furthermore, dedicated databases are available on the internet, linked to a range of analytical programs that facilitate rapid analysis of data (http://pubmlst.org/) and have potential for inter-operability between multiple databases.

Ecological, epidemiological and other distributional studies have benefited from the advent of molecular phylogeny. Techniques such as MLST have facilitated rapid global phylogenetic analysis of individual microbial species. Such molecular methodologies allow testing of hypotheses pertaining to the origins and dispersal of unique populations (Ebach *et al.*, 2003). This facilitates resolution of the relatedness between a given population and its' putative source population, and helps identify an organisms' movements and environmental determinants (Ebach *et al.*, 2003; Godoy *et al.*, 2003; Aanensen and Spratt, 2005).

The current study used MLST to characterise *L. monocytogenes* strains identified as contaminants of an Australian food processing facility. Specifically, the study aimed to use MLST to: (i) differentiate *L. monocytogenes* strains recovered from two food processing factory surveys over a twelve month period, (ii) compare the resolution of MLST and repetitive element PCR, (iii) determine the source of factory contamination by *L. monocytogenes*, and, (iv) determine if MLST can differentiate environmentally persistent and non – persistent (sporadic) *L. monocytogenes* strains.

2.2 Materials and Methods

2.2.1 Factory sampling and isolation of *Listeria* spp. and *Listeria monocytogenes*

A single food processing facility was sampled for *L. monocytogenes* (Ross, Mellefont and Latham, unpublished). Briefly, 131 potentially contaminated sites within the facility were swabbed in May, 2007, and 67 sites in November, 2007. Sites positive for *L. monocytogenes* in the May sampling were swabbed again in November as well as other potentially contaminated sites. Swabbing was conducted aseptically using Nasco Whirlpak-Specisponges (Arrow Scientific, Sydney, Australia) moistened with 25 mL of buffered peptone water (Arrow Scientific, Sydney, Australia). Dry swabs were used for wet sites and buffered peptone water was added after swabbing. Swabs were stored for up to 24 hours at 4°C before being processed for general *Listeria* spp. by Tasmanian Laboratory Services (a nationally accredited, commercial, testing laboratory). This involved 24 hours enrichment in demi-Fraser broth (Oxoid, Australia) incubated at 35°C. A 0.1 mL aliquot of the pre-enrichment broth was transferred into 10 mL of Fraser broth (Oxoid, Australia) and incubated for 48 hours at 37°C. Subculture onto PALCAM (Oxoid, Australia) agar plates followed by incubation at 30°C was performed at 24 and 48 hours. Presumptive *Listeria* spp. were identified on the PALCAM plates and confirmed using the VIDAS *Listeria* species automated assay system (BioMerieux, QLD, Australia). Confirmed *Listeria spp.* isolates were transported to the Food Safety Centre in Hobart, Tasmania, for speciation and strain differentiation.

Confirmatory identification of *L. monocytogenes* was by polymerase chain reaction (PCR) analysis of the listeriolysin O (LLO) gene using the method described

by Border *et al.* (1990). Confirmed *L. monocytogenes* isolates were differentiated by rep-PCR as described previously (Jersek *et al.*, 1999; Rademaker and De Bruijn, 1997) using the REP (Versalovic *et al.*, 1994), ERIC (Versalovic *et al.*, 1991) and BOX (Versalovic *et al.*, 1991) primer sets. LLO PCR and rep-PCR was performed by Mr Roger Latham (Food Microbiology Group, School of Agricultural Science, University of Tasmania).

2.2.2 Strain differentiation by multilocus sequence typing

2.2.2.1 Overview

The relatedness of a subset of the factory *L. monocytogenes* isolates was assessed using the *L. monocytogenes* MLST scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html) developed by Sylvain Brisse, Marie Ragon and Alban le Monnier of the Institut Pasteur, France, adapted from the MLST scheme developed by Maiden *et al.* (1998), and based on software originally developed by Keith Jolley of the University of Oxford, United Kingdom (Jolley *et al.*, 2004). The scheme is based on DNA sequence variation in seven housekeeping loci that have been selected for their slow rate of change (Maiden *et al.*, 1998). An outline of the MLST process is detailed in Figure 2.1. To date, 621 *L. monocytogenes* isolates have been typed, with 320 unique sequence types (ST) identified.

2.2.2.2 Polymerase chain reaction and sequence analysis for MLST

PCR was performed using ImmoMix Red Master Mix (Bioline, Alexandria, Australia) according to manufacturer instructions. Optimal reaction volumes were established and are detailed in Table 2.1. Details of the primers used for PCR amplification and sequencing of the *L. monocytogenes* MLST alleles are outlined in Table 2.2. Amplification was performed on a Peltier PTC-200 thermocycler (MJ Research, Waltham, U.S.A.) using the cycling conditions of four minutes at 94°C followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 52°C and two minutes at 72°C. A final extension step of 10 minutes at 72°C terminated the reaction. These cycling conditions were used for all primer sets except *bglA* which had an annealing temperature of 45°C rather than 52°C.

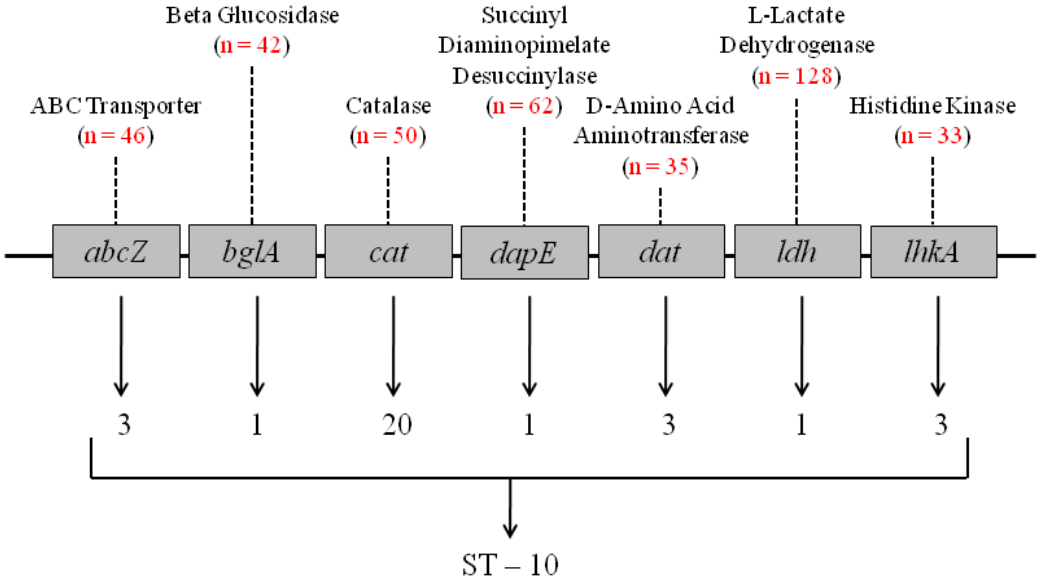


Figure 2.1 The MLST process. Each locus (shaded rectangles) of *L. monocytogenes* used in the MLST scheme has a number of variant alleles (red text) that have been identified and recorded on the MLST database. The sequenced, PCR amplification product of each locus will correspond to either a known or novel allele variant. This will be assigned an existing (or new if novel) integer (between 0 and 46 for the *abcZ* locus for example). The combination of assigned integers (n = 7) is called an allelic profile. The allelic profile will correspond to either a known, or novel, sequence type (sequence type - 10 in this example).

Table 2.1 Optimal reagent concentrations determined for the *L. monocytogenes* MLST PCR reactions. Template concentration varied depending on individual DNA extractions, however, a working amount of 1.5µg in a 50µL reaction produced consistent results.

Reagent	[Stock]	[Working]	Volume
ImmoMix Red Master Mix	n/a	n/a	25µL
Forward Primer	25µM	0.5µM	1µL
Reverse Primer	25µM	0.5µM	1µL
Template DNA	n/a	1.5µg	n/a
Autoclaved Molecular Grade Water[a]	n/a	n/a	To 50µL

[a] 18.2mΩ.

Table 2.2 The primer names (forward: F, reverse: R), sequence, locus and amplicon size used in the MLST PCR scheme for *L. monocytogenes* (Ragon *et al.*, 2008). The portion of primer sequence in bold font represents the universal sequencing tails added to the primers, allowing all of the alleles to be sequenced using the same forward and reverse sequencing primers.

Primer Name	Locus	Primer Sequence	Product Size (bp)
abcZoF	ABC Transporter	5'-**GTTTTCCCAGTCACGACGTTGTA**TCGCTGCTGCCACTTTTATCCA-3'	537
abcZoR	ABC Transporter	5'-**TTGTGAGCGGATAACAATTT**CTCAAGGTCGCCGTTTAGAG-3'	537
bglAoF	Beta-Glucosidase	5'-**GTTTTCCCAGTCACGACGTTGTA**GCCGACTTTTTATGGGGTGGAG-3'	399
bglAoR	Beta-Glucosidase	5'-**TTGTGAGCGGATAACAATTT**CCGATTAAATACGGTGCGGACATA-3'	399
catoF	Catalase	5'-**GTTTTCCCAGTCACGACGTTGTA**ATTGGCGCATTTTGATAGAGA-3'	486
catoR	Catalase	5'-**TTGTGAGCGGATAACAATTT**CAGATTGACGATTCCTGCTTTTG-3'	486
dapEoF	Succinyl-Diaminopimelate Desuccinylase	5'-**GTTTTCCCAGTCACGACGTTGTA**CGACTAATGGGCATGAAGAACAAG-3'	462
dapEoR	Succinyl-Diaminopimelate Desuccinylase	5'-**TTGTGAGCGGATAACAATTT**CATCGAACTATGGGCATTTTTACC-3'	462
datoF	D-Amino Acid Aminotransferase	5'-**GTTTTCCCAGTCACGACGTTGTA**GAAAGAGAAGATGCCACAGTTGA-3'	471
datoR	D-Amino Acid Aminotransferase	5'-**TTGTGAGCGGATAACAATTT**CTGCGTCCATAATACACCATCTTT-3'	471
ldhoF	L-Lactate Dehydrogenase	5'-**GTTTTCCCAGTCACGACGTTGTA**GTATGATTGACATAGATAAAGA-3'	453
ldhoR	L-Lactate Dehydrogenase	5'-**TTGTGAGCGGATAACAATTT**CTATAAATGTCGTTCATACCAT-3'	453
lhkAoF	Histidine Kinase	5'-**GTTTTCCCAGTCACGACGTTGTA**AGAATGCCAACGACGAAACC-3'	480
lhkAoR	Histidine Kinase	5'-**TTGTGAGCGGATAACAATTT**CTGGGAAACATCAGCAATAAAC-3'	480

Following initial amplification, the size of the PCR product was confirmed using agarose gel electrophoresis (Appendix 1.2). The PCR product was purified prior to sequence analysis using a HiYield PCR DNA Extraction Kit (RealBiotech, Taiwan) according to manufacturer instructions. The concentrated DNA was stored at 4°C. A minimum of three separate PCR reactions for each primer set was performed per isolate. The PCR reaction products were divided in two, and three forward and three reverse sequence traces were obtained using the universal sequencing primers US-*F*: 5' – GTT TTC CCA GTC ACG ACG TTG TA – 3' and US-*R*: 5' - TTG TGA GCG GAT AAC AAT TTC. Base sequence analysis of the purified PCR product was performed by Macrogen Inc. of South Korea (http://dna.macrogen.com/). Sequencing reactions were conducted with the universal sequencing primer under BigDye terminator (Applied Biosystems) cycling conditions and run on a 3730xl DNA analyser (http://dna.macrogen.com/).

2.2.2.3 Determination of allelic profiles and sequence type assignment

The sequence data for each primer pair was cleaned and trimmed using Mega (Version 3.1) (Kumar *et al.,* 2004). This allowed removal of primer sequence and highlighted regions where the sequence could reliably be determined. The cleaned sequences (forward and reverse) were then aligned using Mega (Version 3.1) and further trimmed to correspond to the region used to define that allele, based on the allele templates available on the *L. monocytogenes* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/primers_Lmono.html). Multiple correctly trimmed sequences of individual alleles were aligned using Mega (Version 3.1) to identify the actual sequence for that allele, for each *L. monocytogenes* isolate. This was repeated for each of the seven alleles.

Allelic profiles were determined by comparing the sequences against all known alleles using the multiple locus query application of the *L. monocytogenes* MLST scheme (http://www.pasteur.fr/cgi-bin/genopole/PF8/mlstdbnet.pl?page=allseq&file=Lmono_profiles.xml). Complete allelic profiles (seven integers assigned) were compared against all known allelic profiles to determine if the sequence type has previously been described. This was performed using the allelic profile query application of the *L. monocytogenes* MLST scheme (http://www.pasteur.fr/cgi-bin/genopole/PF8/mlstdbnet.pl?page=profile-query&file=Lmono_profiles.xml).

2.2.2.4 Determination of relatedness

Determination of relatedness was achieved using the dedicated MLST data analysis tools available at http://pubmlst.org/analysis/. The level of population relatedness, identified by analysis of the variation between allelic profiles, and individual alleles, was determined for the factory isolates by linkage analysis using LIAN software (Version 3.5). This was performed according to the method first described by Haubold and Hudson (2000), and described in detail by Haubold (2006). Significance was assigned at $p \leq 0.05$.

Cluster analysis (definition of sequence type groups) was performed using the BURST (Based Upon Related Sequence Type) clustering algorithm. The BURST algorithm is based on the matrix of pairwise differences in allelic profiles and unweighted pair group method with arithmetic averages (UPGMA), coupled with conditional bootstrapping. This algorithm was developed by the Department of Infectious Disease Epidemiology at Imperial College, London, United Kingdom (Feil *et al.*, 2004). The method uses a simple model of bacterial evolution in which an ancestral (founding) genotype increases in frequency in the population, and while doing so, begins to diversify to produce a cluster of closely related genotypes that are all descended from the founding genotype. The clusters of related genotypes are referred to as a clonal complex. Finally, phylogenetic analysis was performed using the Phylogeny Inference Package (PHYLIP) (http://evolution.genetics.washington.edu/phylip/general.html).

2.3 Results

2.3.1 Isolation of *Listeria* species and confirmation of *Listeria monocytogenes*

Twenty-nine samples were positive for *Listeria* spp. from the May 2007 factory sampling, with 13 of these confirmed to be *L. monocytogenes* (Table 2.3). The November 2007 factory sampling recovered 34 *Listeria* spp., with 19 of these confirmed to be *L. monocytogenes* (Table 2.4).

2.3.2 Strain differentiation by multilocus sequence typing

Strain differentiation by rep – PCR distributed the *L. monocytogenes* isolates into fourteen groups (Table 2.5). Rep – groups A – F were assessed for relatedness using MLST (Table 2.6). These groups encompassed the persistent contaminants (those strains recovered from both factory surveys), and comprised of the largest number of isolates.

2.3.2.1 Allelic profiles and sequence type assignment

MLST resolved the 14 *L. monocytogenes* strains into five groups (Table 2.7; Appendix 3). Three of these groups are previously described Australian sequence types that have been associated with listeriosis in humans. These include ST – 3 (thirty-two MLST records, isolated from humans between 2001 and 2004), ST – 9 (six MLST records, isolated from humans between 2001 and 2004) and ST – 155 (thirteen MLST records, isolated from humans between 2001 and 2004). ST – 3 has also been identified in Spain (four records associated with human listeriosis from consumption of fish and cheese), as well as the USA and Colombia (one record each, all research strains). ST- 155 also has been identified twice in Russia, isolated from rodents, while ST – 9 has two records from Spain, isolated from cattle. The remaining two groups in the current study are new sequence types, being double and triple locus variants of ST – 204 and ST – 40 respectively.

Strain grouping by MLST did not correlate with the rep – PCR grouping in all instances. MLST highlighted rep – PCR group A and B as members of the rep – PCR groups C (new ST) and D (ST – 3) respectively. Also, strain DS_68, assigned to rep – PCR group D, was observed to be a separate, novel sequence type.

Table 2.3 May 2007 factory survey results. Positive *L. monocytogenes* isolates are presented with their assigned label (DS_*n*), and their corresponding rep – PCR group (bold letter). All sites shown were positive for *Listeria* spp.

Location Description	*L. monocytogenes*
Room A: Freezing Room	
Site 1: Puddle near to a drain.	-
Site 2: Drain lid.	-
Site 3: Cutting board.	-
Site 4: Hopper.	(DS_14) (**A**)
Site 5: Conveyer surface.	(DS_25) (**B**)
Site 6: Puddle next to cutting machine near to a drain.	(DS_31) (**C**)
Site 7: Drain near centre line of room. Lid and surrounding area. Near a cutting machine.	-
Site 7: "Hole" in floor. Large. Near cutting board with trough beneath.	-
Site 8: Puddle. Rear of cutting room. Behind a door and near to a steel and concrete post.	-
Room B: Cutting / Processing Room	
Site 9: Underside of anti-fatigue mat at rear of room. Near to a balance.	(DS_53) (**C**)
Site 10: Drain pipe. Near gloving area.	-
Site 11: Underside of anti-fatigue mat in middle of room. Left middle of room.	-
Room C: Packaging Room	
Site 12: Machine motor housing and feed-belt.	(DS_63) (**C**)
Site 13: Tray. Beneath motor feed-belt.	(DS_68) (**D**)
Site 14: Motorised pallet trolley. Front wheels and fork section.	-
Site 15: Underside of table next to a packaging machine.	(DS_80) (**E**)
Site 16: Switch box of processing machine.	(DS_81) (**D**)
Site 17: Processing machine feed orifice.	(DS_82) (**D**)
Site 18: Machine exit chute guide.	-
Site 19: Holding tray. Feed end.	(DS_84) (**D**)
Site 20: Base of food holding rack frame.	(DS_85) (**D**)
Site 21: Upper surfaces of balance and trolley.	-
Site 22: Upper surface of food holding rack frame.	(DS_88) (**C**)
Site 23: Puddle. Behind black rubber antifatigue mat next to table.	-
Site 24: Puddle. Beside machine feed area.	-
Site 25: Drain. Lid and surround. Between two processing machines.	-
Site 26: Puddle and drain surround. Along wall.	-
Site 27: Wet floor. Adjacent to food weighing trolley.	(DS_PRD_5) (**C**)
Room D: Cutting / Processing Room	
Site 28: Catch tray, beneath machine conveyer, exit side.	-
Site 29: Catch tray, beneath machine conveyer, entry side.	-

Table 2.4 Identification of *L. monocytogenes* from the November 2007 factory survey. Positive *L. monocytogenes* isolates are presented with their assigned label (DS_*n*), and their corresponding rep – PCR group (bold letter). All sites shown were positive for *Listeria* spp.

Location Description	*L. monocytogenes*
Room B: Cutting / Processing Room	
Site 1: Top and bottom of a mat, antifatigue, near to a balance in the middle of the room.	-
Site 2: Underside of mat, antifatigue, beneath a table in the middle of the room.	-
Site 3: Recesses in underside surface of an antifatigue mat. Near to a bench.	-
Site 4: Puddle and crack. Wall-floor corner. Near machine feed end.	(DS_251) (**L**)
Site 5: Floor. Forklift tracking area.	-
Site 6: Drain. Middle of room.	(DS_211) (**G**)
Site 7: Outer door handle. Left end of a chiller.	-
Site 8: Floor. Just outside of a chiller.	-
Site 9: Chiller door seal, damaged. Including insulation.	(DS_219) (**H**)
Site 10: Forklift wheel. Outside the chiller.	(DS_220) (**D**)
Room C: Packaging Room	
Site 11: Food rack. Stainless surfaces.	-
Site 12: Floor. Beneath food rack.	(DS_222) (**E**)
Site 13: Drain-puddle. Rear of room. Close to stairs, sliding door and a dripping pipe.	(DS_268) (**D**)
Site 14: Food table-tray.	(DS_225) (**D**)
Site 15: Drain. Right rear of room.	-
Site 16: Wet floor. Where pallet trolleys run.	(DS_227) (**F**)
Site 17: Mat. Black. Top and bottom. Adjacent to machine.	(DS_232) (**I**)
Site 18: Upper surface of machine conveyer belt.	-
Site 19: Food table. Post processing. Drip accumulation point for machine exit chute.	(DS_236) (**D**)
Site 20: Floor. Beneath food holding table.	(DS_239) (**J**)
Site 21: Underside of mat. Underside. Adjacent to food packing area.	(DS_240) (**K**)
Site 22: Cracked pallet. Near food processing machine.	-
Site 23: Giant Squeegee. Floor contact area. Leaning against wall on right side of room.	(DS_244) (**E**)
Site 24: Castors of trolley-table for post-processed food.	-
Site 25: Gloves. On top of cracked pallet. Near food processing machine.	(DS_247) (**E**)
Site 26: Puddle. Near stair well.	-
Room A: Freezing Room	
Site 27: Drain. Rear right of room.	-
Room D: Processing Room	
Site 28: Floor. Just outside of chiller.	(DS_260) (**M**)
Site 29: Floor puddle. Just in front of chiller.	(DS_261) (**D**)
Site 30: Drain. Just outside of chillers.	-
Site 31: Floor. Centre of wet area in front of food processors.	(DS_264) (**E**)
Site 32: Floor puddle. Immediately in front of food processor.	-
Site 33: Floor. Directly in front of chiller.	(DS_266) (**N**)
Site 34: Food packaging.	**(**DS_B2L**) (F**)

Table 2.5 Rep – PCR groups (A – N) determined for the factory *L. monocytogenes* strains (Latham, 2007). The occurrence and factory distribution (number of rooms each was recovered) of each group identified in each survey is presented.

Group	# Recovered May 2007		# Recovered November 2007	
	# Isolates	# Rooms	# Isolates	# Rooms
A	1	1	0	0
B	1	1	0	0
C	5	3	0	0
D	5	1	5	3
E	1	1	4	1
F	0	0	2	2
G	0	0	1	1
H	0	0	1	1
I	0	0	1	1
J	0	0	1	1
K	0	0	1	1
L	0	0	1	1
M	0	0	1	1
N	0	0	1	1

Table 2.6 The *L. monocytogenes* isolates selected to be assessed for relatedness using MLST. *Persistent contaminants.

Isolate	Rep-Group[a]
DS_14	A
DS_25	B
DS_31	C
DS_53	C
DS_63	C
DS_68	D
DS_80*	E
DS_81*	D
DS_82*	D
DS_84*	D
DS_85*	D
DS_88	C
DS_PRD5	C
DS_B2L	F

[a]Latham, unpublished (2007)

Table 2.7 Allelic profiles and multilocus sequence types assigned to the *L. monocytogenes* isolates tested. Locus variation (LV) is highlighted in bold, red font. *Novel sequence types. **Persistent contaminants.

rep-Group	Isolate	Locus							MLST	Closest Match	LV
		abcZ	*bglA*	*cat*	*dapE*	*dat*	*ldh*	*lhkA*			
A	DS_14	5	**4**	6	4	5	**1**	1	*	ST – 204	Double
B	DS_25	4	4	4	3	2	1	5	3	-	-
C	DS_31	5	**4**	6	4	5	**1**	1	*	ST – 204	Double
C	DS_53	5	**4**	6	4	5	**1**	1	*	ST – 204	Double
C	DS_63	5	**4**	6	4	5	**1**	1	*	ST – 204	Double
D	DS_68	**7**	**4**	**6**	3	2	2	5	*	ST – 40	Triple
E	DS_80**	7	10	16	7	5	2	1	155	-	-
D	DS_81**	4	4	4	3	2	1	5	3	-	-
D	DS_82**	4	4	4	3	2	1	5	3	-	-
D	DS_84**	4	4	4	3	2	1	5	3	-	-
D	DS_85**	4	4	4	3	2	1	5	3	-	-
C	DS_88	5	**4**	6	4	5	1	**1**	*	ST – 204	Double
C	DS_PRD5	5	**4**	6	4	5	1	**1**	*	ST – 204	Double
F	DS_B2L	6	5	6	4	1	4	1	9	-	-

2.3.2.2 Determination of relatedness

No significant difference between the allelic profiles of each sequence type was determined by linkage equilibrium analysis (LIAN Version 3.5; Haubold, 2006; Haubold and Hudson, 2000) (I_A = 0.0456, p >0.05; Table 2.8). The index of association (I_A) is a function of the rate of recombination, and is zero for linkage equilibrium. If the population is in linkage equilibrium, as in this study, it suggests that recombination events predominate over mutation (Haubold and Hudson, 2000). In addition to this, the genetic diversity between individual loci of the strains studied in this work, calculated according to the method described by Haubold (2006), was relatively high (Table 2.9). When coupled with the observed I_A, these results indicate that recombinational exchanges between these *L. monocytogenes* populations were high, and suggest that the populations may be resident to the factory.

Table 2.8 LIAN (Version 3.5) analysis of the multilocus dataset obtained in the current study. The simulation results were obtained from 1000 random resamplings. The simulated significance takes precedence over the parametric, as the data cannot be assumed to be parametric given the minimal ST tested (Haubold and Hudson, 2000).

Quantity	Value
Sequence Types	5
Loci	7
Mean genetic diversity (H)	0.7571 ± 0.0369
Observed mismatch variance (V_D)	1.5667
Expected mismatch variance (V_e)	1.2300
Standardised index of association (I_A)	0.0456
Simulated critical value (5%) ($L_{monte\ Carlo}$)	2.2333
Calculated critical value (5%) ($L_{parametric}$)	2.0873
Simulated significance ($P_{Monte\ Carlo}$)	0.3900
Calculated significance ($L_{Parametric}$)	0.3610

Table 2.9 Genetic diversity (GD), determined for each locus, between each of the sequence types identified in the current study. Zero = no diversity. GD was calculated according to the method described by Haubold (2006)

Locus	GD
abcZ	0.9
bglA	0.7
cat	0.7
dapE	0.8
dat	0.8
ldh	0.8
lhkA	0.6

BURST analysis mirrored the group clustering determined by phylogenetic analysis in all instances. Phylogenetic analysis of the factory *L. monocytogenes* strains was performed under the assumption that the populations were resident, descending from a common ancestor (Figure 2.2). With this in mind, the analysis suggested that ST – 3 and the new ST assigned to isolate DS_68 descended from a common ancestor that had branched from the predicted founding member. Similarly, ST – 9 and the new ST assigned to strain DS_14 and other members descended from a common ancestor that branched from ST – 155, which originated by branching from the predicted founding member.

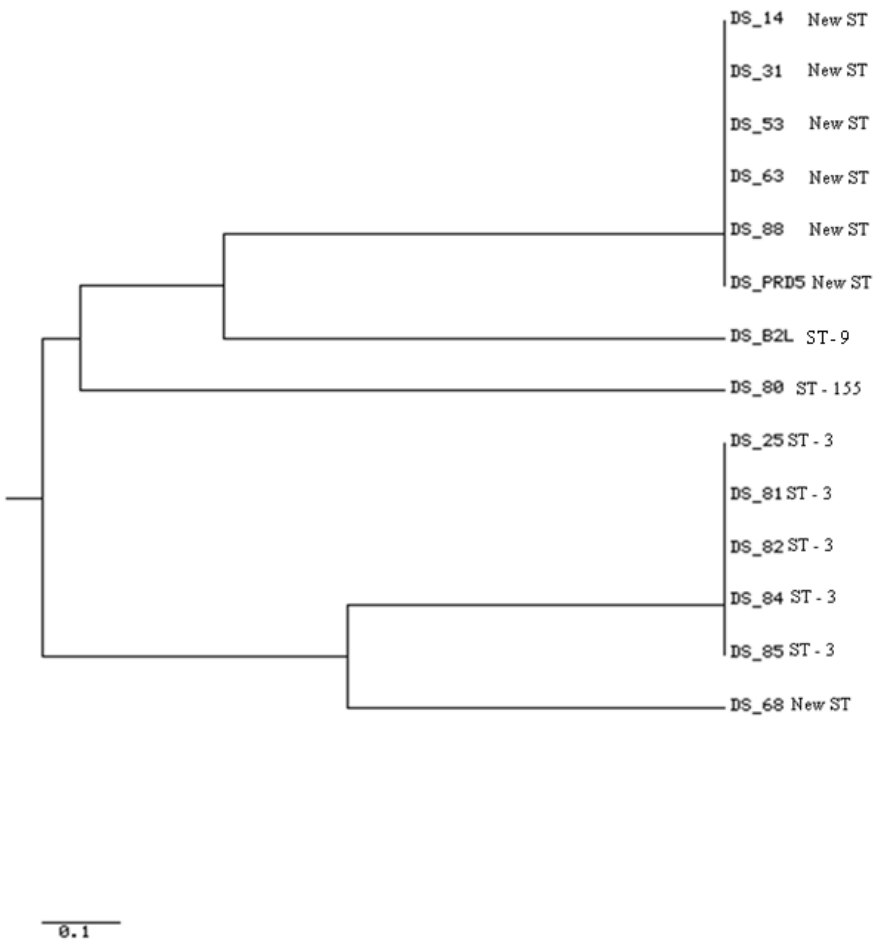


Figure 2.2 UPGMA tree predicting the phylogeny of the factory *L. monocytogenes* isolates.

Phylogenetic analysis of all known Australian ST's, including the factory *L. monocytogenes* strains resolved in the current study, identified an evolutionary hierarchy dictated by the sequence type, serotype and lineage of the *L. monocytogenes* strain (Figure 2.3). Furthermore, this allowed prediction of serotype and lineage for those factory strains that corresponded to previously known ST's, and prediction of lineage for the newly identified ST's.

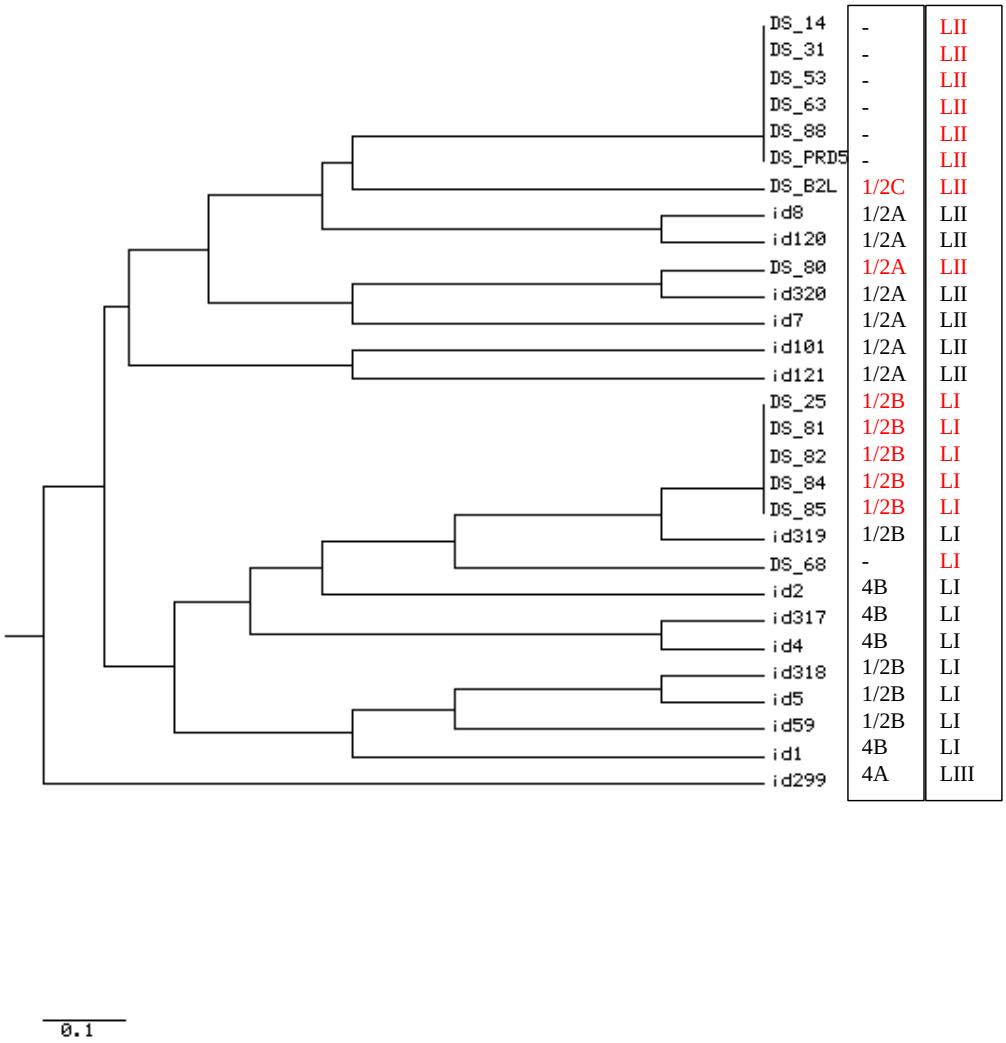


Figure 2.3 UPGMA tree comparing the phylogeny of all known Australian *L. monocytogenes* ST's. Strain DS_B2L is ST – 9, DS_80 is ST – 155, and the cluster incorporating DS_25 – DS_85 are ST – 3. These have all been identified previously. Serotype and lineage were assigned if known, or inferred in the case of the factory strains with previously defined ST's. Lineage was predicted for the novel ST's based on their position on the UPGMA tree (red font).

2.4 Discussion

In this study, MLST resolved 14 *L. monocytogenes* strains recovered from two factory surveys into five distinct groups. Furthermore, MLST improved on the discriminatory power of the rep – PCR typing scheme previously applied to this strain set, separating one rep – PCR group and removing another all together. Rep – PCR is an established, robust method for strain differentiation in *L. monocytogenes*, and has been used in a number of studies (Versalovic *et al.*, 1991; Versalovic *et al.*, 1994; Jersek *et al.*, 1999 and others). That method measures rapidly evolving genomic variation rather than the slowly evolving variation observed through MLST (Maiden *et al.*, 1998). However, it is unlikely that this could explain the variation

observed between rep - PCR and MLST in this study. In typing the current strain set using rep – PCR, visual differentiation of agarose gel electrophoresis patterns was applied, rather than one of the computer assisted differentiation systems available (Rademaker *et al.,* 2000). The increased resolution obtained by MLST, although anticipated to some extent due to its nucleotide basis, may, in part, reflect the subjective nature of visual differentiation of rep – PCR groups. On this premise, future works utilising rep – PCR as a strain differentiation method should, as recommended by Rademaker *et al.* (2000), employ a computer assisted differentiation system to limit observational discrepancies.

Of the five MLST's identified in the current work, three corresponded to previously described ST's, with each of these recovered from human infections on multiple occasions. These sequence types (ST – 3, 9 and 155) have numerous entries on the MLST database corresponding to serotypes 1/2a (ST – 155), 1/2b (ST – 3) and 1/2c (ST – 9). On this basis, it seems that these serotypes correspond to these sequence types, and suggests that MLST may be an effective, indirect determinant of strain serotype. Notably, serotype 1/2a and 1/2b strains of *L. monocytogenes* are associated (along with serotype 4b) with the majority of human *L. monocytogenes* infections (Lukinmaa *et al.,* 2003). Given that these strains were recovered from a food processing environment, and that ST – 3 was a persistent contaminant of these facilities based on the surveys conducted, these strains potentially constitute very high risk *L. monocytogenes* populations, and may represent a significant risk to public health.

Numerous studies have shown that it is possible to use molecular methodologies to determine the route of *L. monocytogenes* contamination within factory environments (e.g. Alessandria *et al.*, 2010). The relatedness of each of the MLST's identified in this study implied that they may have resided, and been dispersed throughout, the food processing facility for a prolonged period of time. Further to this, the distribution of MLST's throughout the factory, including multiple rooms and equipments, suggest that the strains are being actively disseminated throughout the factory. This confounded identification of a specific reservoir, and suggested a self perpetuating, rather than external, contamination source. Active dissemination by daily factory processes appears likely, evidenced by the presence of *L. monocytogenes* ST's on the wheels of forklifts, on moveable trolleys, and in water puddles located in walk ways. Given these and other locations where *L. monocytogenes* was recovered, including post processing food racks, it seems

highly probable that *L. monocytogenes* from this food facility will, or has been, transferred into the food supply chain. This is of serious concern and may be indicative of a systemic microbial control failure within the facility.

The current study compared the sequence types of the factory isolates against all previously known Australian ST's. To date, eighteen MLST's have been assigned to Australian *L. monocytogenes* isolates, encompassing 109 isolates. As already stated, three of the factory ST's corresponded to known Australian ST's, with the remaining two not previously described. Phylogenetic comparison showed that all of the ST's, including those recovered from the factory, clustered according to *L. monocytogenes* strain serotype and lineage. This was presented as three main tree arms corresponding to lineage I, II and III strains, with branching within these arms correlated to serotypes characteristic of the lineage. This permitted inferential assignment of lineage and serotype to those factory *L. monocytogenes* strains corresponding to known ST's, and allowed prediction of likely lineages for the strains corresponding to novel ST's. However, given that there appears to be a relational bias corresponding to lineage and serotype, the phylogenetic relationship of the survey factory *L. monocytogenes* isolates must be discounted, as this may simply represent evolutionary descent for the species as a whole.

Lineage I strains are significantly more common from human sources, while lineage II strains are significantly more common from food sources, and lineage III isolates are rarely recovered from either food or humans (Gray *et al.*, 2004). Sequence types corresponding to two of these lineages, however, were recovered from the factory survey, including ST – 3 (lineage I), ST – 9 (lineage II) and ST – 155 (lineage II). These results highlight the presence of high risk *L. monocytogenes* strains within the survey factory.

The use of MLST as a means of identifying high risk sub – populations of microbes within complex environments is not new. The method is regularly employed in epidemiological investigations / studies, as well as works investigating the prevalence and distribution of specific pathogen subtypes within environments, such a multi – drug resistant *Escherichia coli* (Simoes *et al.*, 2010; Urwin and Maiden, 2003). At present, MLST of *L. monocytogenes* has largely been focused on isolates of clinical origin. Given that *L. monocytogenes* has an environmental reservoir, knowledge of the distribution of this organism in both natural and anthropogenic settings could substantially aid investigations on its movements,

microecology and infection foci, and help to guide the logic of prevention and intervention strategies.

In the present study, MLST identified high risk *L. monocytogenes* strains associated with persistent contamination of a food processing factory. However, in some instances, sporadic and persistent contaminants belonged to the same sequence type (e.g. DS_81 and DS_25). Although MLST has been shown capable of identifying high – risk *L. monocytogenes* populations in this study, knowledge of the factors underpinning the incongruent distribution of environmentally persistent strains is also of considerable importance. The acquisition of persistent *L. monocytogenes* strains presents an opportunity to study environmental persistence in this species in greater detail. Further work using the environmentally persistent and sporadic strains from this study to investigate factors that may contribute to environmental persistence, such as biofilm production and physiological stress, is required. This could improve knowledge of the mechanisms affording environmental persistence in *L. monocytogenes* and help guide preventative strategies directed at this organism. This aim forms the basis of the following experimental chapters of this thesis.

CHAPTER 3
ENVIRONMENTAL AND STRAIN SPECIFIC INFLUENCES ON BIOFILM FORMATION BY *LISTERIA MONOCYTOGENES*

3.1 Introduction

The development of microbial biofilms on surfaces associated with food production, processing and preservation is a serious concern for the food industry. A biofilm can be defined as a structured community of microbes embedded within an organic polymeric matrix that is irreversibly adhered to a surface (Donlan, 2002). Any wet inorganic or organic surface is suitable for the establishment of a biofilm, and it is accepted that this mode of microbial existence is much more prevalent in nature than a free living planktonic state (Costerton *et al.,* 1995, Davey and O'Toole, 2000).

Conceptual models describe the establishment of a microbial biofilm as a complex, but ordered, sequence of events. A number of models have been developed to illustrate this process. A simple six stage model for the formation of a biofilm is succinctly described by Allison and Gilbert (1993), and will be used here to provide an overview of the process. The six stages include:

i. Conditioning of the substratum.
ii. Reversible attachment of the microorganism.
iii. Irreversible attachment of the microorganism.
iv. Formation of microcolonies and matrix deposition.
v. Colony stratification.
vi. Detachment and dispersal of biofilm members.

Each stage of this process may differ to some extent depending on the microbial genera, species, attachment surface, environmental conditions and physiological status of the microorganism. However, the model defines the generalised sequence of events involved in microbial biofilm formation. Details of Allison and Gilbert's (1993) biofilm formation model are illustrated in Figure 3.1.

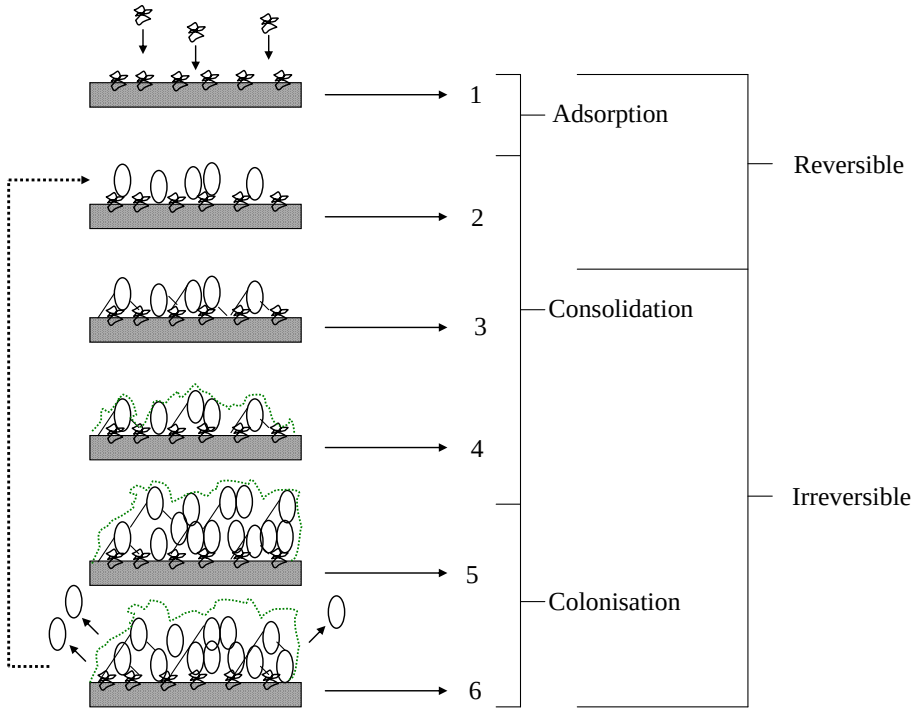


Figure 3.1 A simplified overview of the sequence of events involved in the establishment of a microbial biofilm (adapted from Allison and Gilbert (1993)). **Stage 1** involves conditioning of the attachment substrate by adsorption of organic molecules. This occurs naturally by molecular diffusion or mass transfer. **Stage 2** incorporates initial attachment of microbes to the substratum. This is a critical stage of biofilm development, initiating biofilm consolidation, and is influenced by microbe motility, environmental conditions and other extrinsic and intrinsic factors. **Stage 3** marks the transition to a biofilm proper. Bacterial attachment is now irreversible, facilitated by surface appendages such as pili, flagella and fimbriae. **Stage 4** involves division and development of the primary colonising cells to form microcolonies, along with deposition of matrix through production / secretion of exopolysaccharide and other organic molecules. **Stage 5** marks the transition to a mature biofilm. Growth continues and stratification of microbial cells occurs through to the final thickness, architecture and metabolic activity of the biofilm. This is variable, depending on the microbial genera / species, attachment surface, environmental conditions and physiological status of the microorganism. Finally, **Stage 6** is characterised by sloughing and dispersal of cells from the biofilm. This occurs through a number of physical and intrinsic forces, such as shear forces and death of cells within the deeper biofilm strata. The sloughed cells may go on to develop new biofilms, perpetuating the cycle.

Importantly, biofilms form a protective microbial environment while permitting essential transfer processes to occur with the outside environment. This is problematic to food industries as these facilities may provide optimal conditions for the establishment of biofilms, which, once established, may act as reservoirs of microbes with increased resistance or even refraction to commonly used cleansing and sanitising agents (Costerton *et al.*, 1995; Pan *et al.*, 2006). Of particular concern are reports that exposure to the cleaning regimes can enhance intrinsic resistance to these agents within certain microbial populations, and an association with increased

virulence has been described (Kastbjerg *et al.,* 2009). If uncontrolled, the biofilm reservoir can facilitate the dissemination of bacteria throughout the food processing / production environment and food itself, possibly leading to persistent contamination. Furthermore, it may lead to augmentation, production and development of physiologically "toughened", potentially virulent, bacteria throughout these environments, resulting in an increased risk to public health (Begley *et al.,* 2009; Kastbjerg *et al.,* 2009).

Listeria monocytogenes is a common contaminant of food processing and production plants, and persistent environmental contamination and biofilm formation by this serious food-borne pathogen are well described (Moretro and Langsrud, 2004; Ho *et al.,* 2007; Lunden, 2002). Biofilms produced by *L. monocytogenes* are architecturally simple in comparison to those of many other microorganisms, but their contribution to resistance and environmental persistence is widely reported (Rieu *et al.,* 2008; Borucki *et al.,* 2003; Chae and Schraft, 2000; Harvey *et al.,* 2007; Kalmokoff *et al.,* 2001; Moretro and Langsrud, 2004).

Adhesion by *L. monocytogenes* is arguably the most critical stage of biofilm development for this species, and strain differences in adhesion have been demonstrated (Borucki *et al.,* 2003; Chae and Schraft, 2000; Harvey *et al.,* 2007; Kalmokoff *et al.,* 2001; Moretro and Langsrud, 2004). There are many contrasting hypotheses on the reasons for these differences. Environmentally persistent phenotypes, for example, have been described as having increased adhesive ability, while others report no correlation between environmental persistence and biofilm formation (Borucki *et al.,* 2003; Djordevic *et al.,* 2002; Lunden *et al.,* 2000). Similar conflicting reports on lineage and serotype are available (Chae *et al.,* 2006; Kalmokoff *et al.,* 2001). Harvey *et al.* (2007) attempted to resolve this issue in a study of 138 well characterised *L. monocytogenes* isolates. Despite identifying esterase electrophoresis type II isolates as prolific biofilm formers, variable biofilm production was still observed within this group and, ultimately, no definitive relationships were discerned.

Environmental stresses such as nutrient status, temperature and pH have been suggested to influence adhesion and biofilm development in *L. monocytogenes* (Begley *et al.,* 2009; Djordevic *et al.,* 2002; Norwood and Gilmour, 2001). Flagellar motility, autoinducer mediated communication and cell surface structures, such as the internalins, appear to have a role; however the intrinsic "strain specific" mechanisms remain to be *definitively* characterised (Lemon *et al.,* 2007; Sela *et al.,*

2006). While *L. monocytogenes* strains certainly vary in their ability to attach to substrates and form biofilms, much remains to be learned about the underlying reasons for these differences. Such knowledge is essential to develop effective preventative measures and reduce the public health risk and economic burden *L. monocytogenes* presents to the food industry and public.

This study aimed at increasing the understanding of *L. monocytogenes* biofilm formation, by elucidating intrinsic and extrinsic factors that influence their development, to help guide preventative measures and contribute to the development of targeted *L. monocytogenes* biofilm controls. Specifically, 95 *L. monocytogenes* isolates, of diverse serotype and origins, were assembled to (i) assess the effect of high and low incubation temperatures on *L. monocytogenes* strains biofilm formation and identify subgroups among those strains with increased propensity to form biofilms, (ii) assess the effect of acidic and alkaline pH on *L. monocytogenes* strains biofilm formation and identify subgroups with increased propensity to form biofilm, (iii) determine if the serotype or source of isolation of the *L. monocytogenes* strain has any influence on biofilm formation at different temperature and pH, and (iv) determine if environmentally persistent *L. monocytogenes* strains produce more biofilm than sporadic strains (strains recovered only once over a sampling period) at different temperatures and pH.

3.2 Materials and Methods

3.2.1 Overview

Biofilm formation by 95 strains of *L. monocytogenes* at different temperatures was assessed. Biofilm formation by a subset of 16 strains, including 14 strains isolated from a single Australian food processing facility (Chapter 2) was assessed at different pH levels. Finally, biofilm production under acidic and alkaline growth conditions by both a persistent and a sporadic strain from the factory subset was observed using scanning electron microscopy.

3.2.2 Bacterial strains

Ninety-five *L. monocytogenes* isolates obtained from environmental, animal, food and clinical sources were studied. Environmentally persistent (recovered more than once from the same environment over a sampling period) and sporadic (recovered only once from the same environment over a sampling period) strains were represented. Details of all strains are outlined in Table 3.1.

Table 3.1 Summary table of the *Listeria monocytogenes* strains used in the present study.

Strain	Nature	Origin	Serotype
1. L1 (S2542)[a]	Environmental	Smoked Salmon	1/2a
2. 102-695-S1-154[a]*†	Environmental	FPP	1/2a
3. Joyce[a]	Unknown	Ovine	1/2a
4. FW04-25[a,b]	Unknown	Food Product	1/2a
5. 87-1599[a]	Unknown	Bovine	1/2a
6. 102-265-S3-352[a]*†	Environmental	FPP + Product	1/2a
7. Liver[a]	Clinical	Liver tissue	1/2a
8. FW04-19[a]	Unknown	Human	1/2b
9. 114-997-S7-63[a]*†	Environmental	Food Product	1/2b
10. L2 (S2657)[a]	Environmental	Smoked Salmon	1/2b
11. FW04-17[a]	Unknown	Clinical	1/2b
12. 102-195-S1-242[a*]†	Environmental	FPP + Product	1/2c
13. LO28[a]	Unknown	Human	1/2c
14. FW03-32[a]	Unknown	Food Product	3a
15. 102-265-S3-745[a*]	Environmental	FPP + Product	3a
16. ATCC 19114[a,b]	Clinical	Bovine	4a
17. ScottA[a]	Clinical	Human	4b
18. ATCC 19115[a]	Clinical	Human	4b
19. 102-241-S1-349[a]*†	Environmental	Food Product	4b
20. FW03-35[a]	Unknown	Food Product	4b
21. FW04-20[a]	Unknown	Clinical	4b
22. 102-231-S7-566[a]*†	Environmental	Food Product	4b
23. FW04-21[a]	Unknown	Clinical	4b
24. 102-231-S7-232[a]*†	Environmental	Food Product	4b
25. 70-1700[a]	Unknown	Ovine	4e
26. 64-1495[a]	Unknown	Ovine	1/2a
27. FW06-22[a]	Clinical	Human	1/2.
28. 69-1793[a]	Unknown	Bovine	4b
29. 69-577[a]	Unknown	Bovine	4b
30. FW06-17[a]	Clinical	Human	1/2.
31. 83-2795[a]	Unknown	Ovine	4a

32.	FW06-19[a]	Clinical	Human	1/2.
33.	FW06-23[a]	Clinical	Human	4/b
34.	FW06-20[a]	Clinical	Human	1/2.
35.	62-2853[a]	Unknown	Bovine	1/2a
36.	70-0421[a]	Clinical	Wallaby	1/2b
37.	FW06-11[a]	Clinical	Human	1/2.
38.	FW06-34[a]	Clinical	Human	1/2.
39.	FW06-35[a]	Clinical	Human	4b
40.	FW06-40[a]	Clinical	Human	1/2.
41.	FW06-25[a]	Clinical	Human	1/2.
42.	FW06-12[a]	Clinical	Human	1/2.
43.	FW06-50[a]	Clinical	Human	1/2.
44.	FW06-7[a]	Clinical	Human	1/2a
45.	FW03-37[a]	Clinical	Human	1/2a
46.	FW06-13[a]	Clinical	Human	1/2.
47.	FW06-9[a]	Clinical	Human	-
48.	FW06-41[a]	Clinical	Human	1/2.
49.	FW06-38[a]	Clinical	Human	4b
50.	FW06-39[a]	Clinical	Human	1/2.
51.	FW06-16[a]	Clinical	Human	3
52.	FW06-24[a]	Clinical	Human	4b
53.	FW06-15[a]	Clinical	Human	1/2.
54.	FW06-36[a]	Clinical	Human	1/2.
55.	FW06-14[a]	Clinical	Human	-
56.	FW06-46[a]	Clinical	Human	4e
57.	FW06-8[a]	Clinical	Human	1/2.
58.	FW06-47[a]	Clinical	Human	1/2.
59.	FW06-10[a]	Clinical	Human	1/2.
60.	FW06-21[a]	Clinical	Human	1/2.
61.	FW06-18[a]	Clinical	Human	1/2.
62.	FW06-31[a]	Clinical	Human	1/2.
63.	FW06-30[a]	Clinical	Human	1/2.
64.	FW06-29[a]	Clinical	Human	1/2.
65.	FW06-27[a]	Clinical	Human	3
66.	FW06-28[a]	Clinical	Human	1/2.
67.	FW06-26[a]	Clinical	Human	1/2.
68.	FW06-33[a]	Clinical	Human	1/2.
69.	FW06-43[a]	Clinical	Human	3
70.	FW06-45[a]	Clinical	Human	1/2.
71.	FW06-44[a]	Clinical	Human	4b
72.	FW06-32[a]	Clinical	Human	1/2.
73.	FW06-49[a]	Clinical	Human	3
74.	FW06-5[a]	Clinical	Human	4b
75.	FW06-42[a]	Clinical	Human	4b
76.	FW06-6[a]	Clinical	Human	1/2.

77.	FW06-1[a]	Clinical	Human	1/2.
78.	FW06-48[a]	Clinical	Human	3
79.	FW06-4[a]	Clinical	Human	1/2.
80.	FW06-2[a]	Clinical	Human	1/2.
81.	FW06-3[a]	Clinical	Human	3
82.	DS_14[a,b,<]	Environmental	FPP	-
83.	DS_85[a,b,<]	Environmental	FPP	-
84.	DS_25[a,b,<]	Environmental	FPP	-
85.	DS_88[a,b,<]	Environmental	FPP	-
86.	DS_31[a,b,<]	Environmental	FPP	-
87.	DS_PRD5[a,b,<]	Environmental	FPP	-
88.	DS_53[a,b,<]	Environmental	FPP	-
89.	DS_B2L[a,b,∗,<]	Environmental	FPP	-
90.	DS_63[a,b,<]	Environmental	FPP	-
91.	DS_68[a,b,∗,<]	Environmental	FPP	-
92.	DS_80[a,b,∗,<]	Environmental	FPP	-
93.	DS_81[a,b,∗,<]	Environmental	FPP	-
94.	DS_82[a,b,<]	Environmental	FPP	-
95.	DS_84[a,b,<]	Environmental	FPP	-

a Assessed for ability to form biofilm under different temperatures.
b Assessed for ability to form biofilm under different pH environments.
***** This strain was recovered more than once from the same sampling site over a minimum of 12 months and was defined as persistent.
† This strain was kindly provided by Dr John Holah and colleagues from the Campden and Chorleywood Food Research Association, Gloucestershire, United Kingdom.
FPP – Food production plant.
< These strains were all recovered from a single Australian food processing facility

3.2.3 Microtitre plate biofilm production assay

Biofilm production was measured in triplicate for each *L. monocytogenes* isolate using the colourimetric 96 – well microtitre plate method described by Djordevic *et al.* (2002). All isolates were recovered from storage at - 80°C (Appendix 1.1), inoculated onto brain – heart infusion (BHI) agar (CM225 'BHI'; Oxoid, Australia; Appendix 2.1), and incubated at 25°C for 24 h. The isolates were sub-cultured twice under the same growth conditions to acclimatise them to the growth conditions, after which they were transferred by sterile loop into 10 mL BHI broths (pH 7.3 ± 0.1; Oxoid, Australia; Appendix 2.2) and incubated at 25°C for 24 h. The broth cultures were also sub-cultured twice under the same growth conditions by transferring 100 µL into fresh 9.9 mL BHI broths. Finally, a 100 µL inoculum of each broth culture was added to fresh 9.9 mL BHI broths and gently mixed. A microtitre plate assay was set up as presented in Figure 3.2, and the plates incubated at either 10°C, 20°C, 25°C or 37°C (± 1°C). Biofilm production was measured at 24 and 48 hours (120 and 144 hours for the 10°C treatment).

A subset of 16 *L. monocytogenes* isolates (14 environmentally persistent and sporadic strains recovered from a single Australian food processing facility, and two controls recovered from different sources, and representing different serotypes; a serotype 4a clinical isolate, American Type Culture Collection strain 19114, and a serotype 1/2a isolate recovered from a food product, strain FW04-0025) was assessed for biofilm production under different pH culture conditions at 25° C (Table 3.1). Recovery and culture of the isolates was as previously described (Section 3.2.3) with the following alterations. Four pH treatments were tested (pH 4.7, 5.7, 7.3 and 8.5 ± 0.1). The pH was adjusted through addition of concentrated HCl / NaOH (Sigma – Aldrich, Australia) and measured using an Orion 250A pH meter (Orion Research Inc., USA). Biofilm formation was measured at 24 and 48 hours.

For all treatments, triplicate 200 µL aliquots from each treatment broth were transferred to 96 – well polyvinyl chloride (PVC) microtitre plates (Greiner Scientific, Australia). A row of control wells containing 200 µL of sterile BHI broth was also added. An example of the plate layout is detailed in Figure 3.2. The plates were lidded, sealed using parafilm (Bacto laboratories, Sydney, Australia), and incubated according to the desired experimental treatment. The production of biofilm was measured spectrophotometrically (absorbance at 595 nm) using a Benchmark microplate reader (BioRad, USA). In order to correct for background staining, the absorbance reading of the control wells was deducted from the absorbance readings of the sample wells. Absorbance data was displayed using Microplate Manager® III Macintosh Data Analysis Software (BioRad, USA). Results were presented as the mean and standard deviation of the three replicates, calculated using Microsoft Excel® software. Statistical analysis was performed using SAS statistical software (Version 9.1.2).

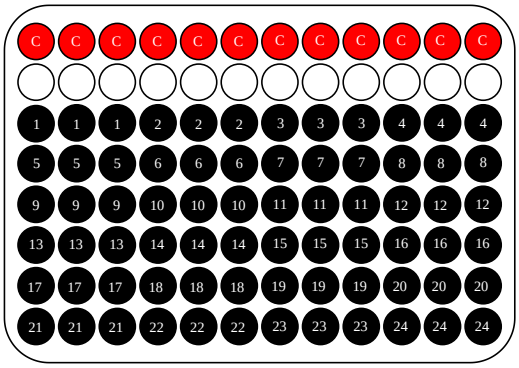


Figure 3.2 96 – well microtitre plate layout used for measurement of biofilm production. The red wells (C) represent control wells filled with 200µL of uninoculated BHI broth. These are followed by a row of empty wells. The black wells represent the samples. Four isolates (× 3 repeats) were added per row for a total of 24 isolates per plate.

The significance of differences in the amount of biofilm measured for each of the *L. monocytogenes* strains under each test condition, and as a function of the nature, origin, serotype, and environmental persistence were assessed by Univariate Analysis of Variance (ANOVA). Significance was assigned at $p \leq 0.05$. Post hoc testing (*t*-test least significant difference, *t*-LSD) was performed to identify significant differences within these qualitative groups. Significance was assigned at $p \leq 0.05$.

Finally, Principal Component Analysis (PCA) was performed to determine which of the test components had the greatest influence on biofilm production. The PCA was based on a correlation matrix as described by Quinn and Keough (2002). The principle components analysed were time point (24 and 48 hours; 120 and 144 hours for the 10°C treatment), and temperature treatment (10, 20, 25 and 37°C) resulting in a total of 8 components. Correlation was considered significant at $p \leq 0.01$ (df = 92; $r \geq 0.264$) due to the large sample set. Components with eigenvalues >1 and those above the elbow of individual scree diagrams were assumed to explain most of the variation (the principle components) within the analysis (Quinn and Keough, 2002).

3.2.4 Scanning electron microscopy

Biofilm establishment of an environmentally persistent and sporadic *L. monocytogenes* isolate recovered from a food processing facility (Chapter 2; isolates DS_14; non-persistent, and DS_81; persistent; Table 3.1) under conditions of pH stress was assessed using scanning electron microscopy (SEM). Three pH treatments were tested (pH 4.7, 7.3 and 8.5 ± 0.1). The pH was adjusted as previously described (Section 3.2.3). All cultures were grown in BHI broth containing a food grade stainless steel coupon (20 mm × 20 mm × 1 mm) and incubated at 25° C for 72 hours. The coupons were preconditioned in BHI broth at 25° C for 24 hours prior to inoculation in fresh BHI broth (with pH adjusted accordingly).

After incubation, the stainless steel coupons were removed from the media and rinsed 5 times using 10 mL of 1 × phosphate buffered saline (pH 7.4; Appendix 1.6). The coupons were placed into 0.2 M cacodylate buffer (pH 7.4; Appendix 1.7) containing 25mL/L glutaraldehyde (Proscitech, Brisbane, Australia) for 24 hours for primary fixation. Following primary fixation, the coupons were given two 5-min

washes in 10 mL of cacodylate buffer supplemented with 6% sucrose and 0.1% calcium chloride (pH 7.4; Appendix 1.8) and placed into 1% osmium tetroxide (Appendix 1.5) for 20 minutes. The coupons then underwent a graded series of ethanol washes (15 minutes each at 40, 50, 70, 90 and 100% ethanol). Immediately after the final wash, the coupons were freeze-dried using a vacuum freeze dryer (Dynavac, U.S.A.) for 5 hours. The dried coupons were sputter coated with gold using a BalTec SCD 050 sputter coater, and viewed with an FEI Quanta 600 MLA environmental scanning electron microscope operated at an accelerating voltage of 15kV.

3.3 Results

3.3.1 Microtitre plate biofilm production assay

3.3.1.1 Microtitre plate biofilm production assay – effect of temperature

Biofilm production increased with incubation time for the 20 and 25°C temperature treatments, and remained essentially static for the 10 and 37°C temperature treatments (Figure 3.3; Appendix 4). This was also observed as temperature increased, although the pattern was not as pronounced as observed for incubation time, with considerable overlap between temperature treatments. This was most evident for those strains that produced lower biofilm levels, and in the 10, 20 and 25°C treatments. Among the most prolific biofilm producing strains, the level of production correlated with both increasing temperature and increasing incubation time: (37°C (Time 2) > 37°C (Time 1) > 25°C (Time 2) > 25°C (Time 1) = 20°C (Time 2) > 20°C (Time 1) = 10°C (Time 2) > 10°C (Time 1)).

Biofilm production among all *L. monocytogenes* strains at time 1 and time 2 are detailed in Appendix 4. From this, strains 102-695-S1-154, 102-231-S7-232, 102-231-S7-566, Joyce and FW03-35 were identified as low biofilm producers, while strains FW06-4, FW06-1, FW06-39, FW06-31 and FW06-8 were identified as high biofilm producers, in all temperature treatments for both incubation times. In addition some groups of strains (e.g. DS_82 – DS_95, *see* Table 3.1) appeared capable of producing more biofilm more quickly than others, dominating the upper measurements at time 1. Biofilm production at time 2 was more moderate for this group; however it was still high in comparison with other strains.

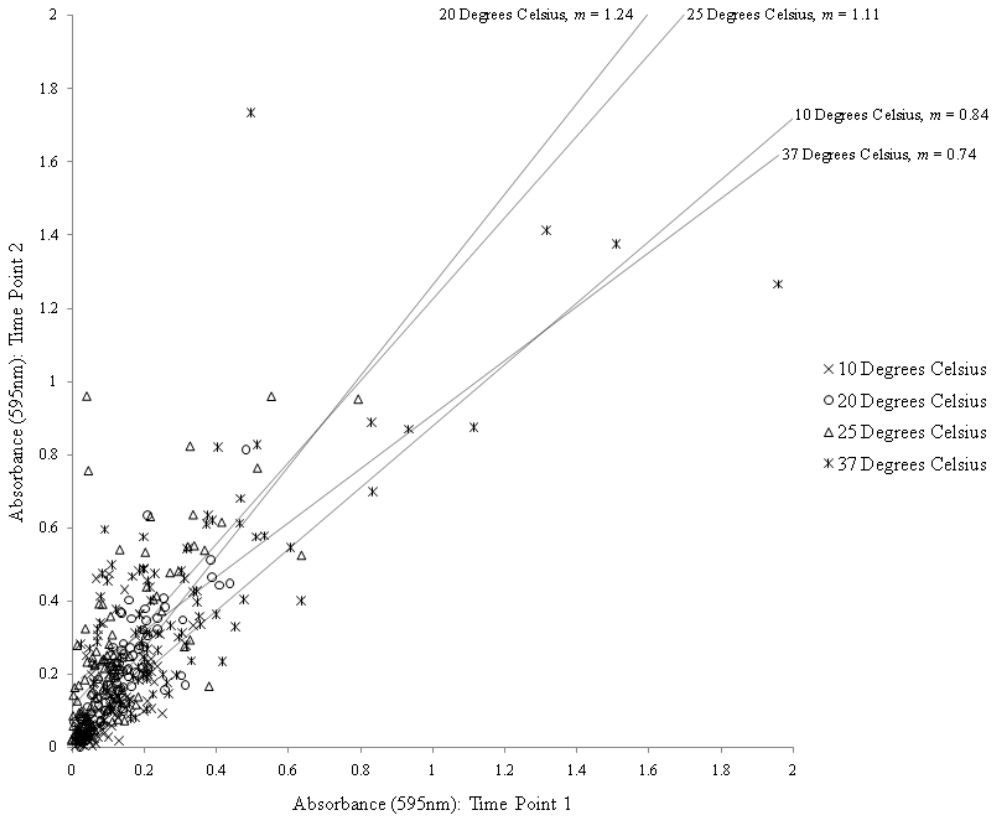


Figure 3.3. Scatterplot of biofilm production at time point 1 (24 h for the 20, 25 and 37°C treatments; 120 h for the 10°C treatment) versus time point 2 (48 h for the 20, 25 and 37°C treatments; 144 h for the 10°C treatment). Points represent the mean of three absorbance measurements for each time point at 595nm. Lines-of-best fit were fitted and the slope (*m*) for each presented.

There were distinct differences in the effect of temperature on biofilm production in sub-groups of *L. monocytogenes* strains examined. All DS_*n* strains, for example, were among the top 20% of biofilm producers at 10°C after 120 h, with most remaining in or near to this range at 144 h. However, for all other temperature treatments, this group was consistently observed to produce only moderate amounts of biofilm. Similarly, the greatest biofilm production in the higher temperature treatments (20, 25 and 37°C) was found among members of the FW-*n* group at both incubation times. These strains were recovered from environmental (a single food production plant) and clinical (human / animal infections) sources, respectively. The clinical FW-*n* group dominated the high biofilm production measurements under most conditions. The DS_*n* group, however, were the most prolific biofilm producers at time 1 (10°C) and also were highly represented at time 2 (10°C).

Principle component analysis was applied to the data to identify the temperature treatment at which biofilm production varied most, and Univariate

ANOVA was used to assess similarity among strains according to the qualitative parameters (nature, origin, serotype and environmental persistence status) used to characterise the *L. monocytogenes* isolates. A scree plot highlighted biofilm production for the 10°C treatment at both time point 1 and 2 as varying most from the other temperature treatments (eigenvalue > 1; Figure 3.4). The correlation matrix resolved this further, confirming that the 10°C treatment at time 2 did not significantly correlate with the 37°C treatments at both time 1 and time 2, and identified this temperature and incubation time as the principle component contributing greatest variation to the analysis. A significant correlation was observed between all other temperature treatments (df = 92, $r \geq 0.264$, $p < 0.01$; Table 3.2).

The greatest variation in biofilm production was observed between the lowest and highest temperature treatments (Table 3.2). Correlation in biofilm production between temperature treatments and incubation times was weak at low temperatures, moderate to strong at 20 and 25°C, weak at time 1 for the 37°C treatment, and moderate to high at 37°C time 2 (Table 3.2). For the 10°C treatments, strong correlation in biofilm production was only observed between time points, with decreasing correlation observed as incubation temperature increased (Table 3.2). In contrast, the highest correlation observed for the 37°C treatment at measurement time 1 was with the 25°C treatment at measurement time 2, however this still remained moderate. Overall, the intermediate temperature treatments (20 and 25°C) showed the least variability and stronger correlations overall. Increasing variation occurred with more extreme temperature, particularly at 10°C (Figure 3.4).

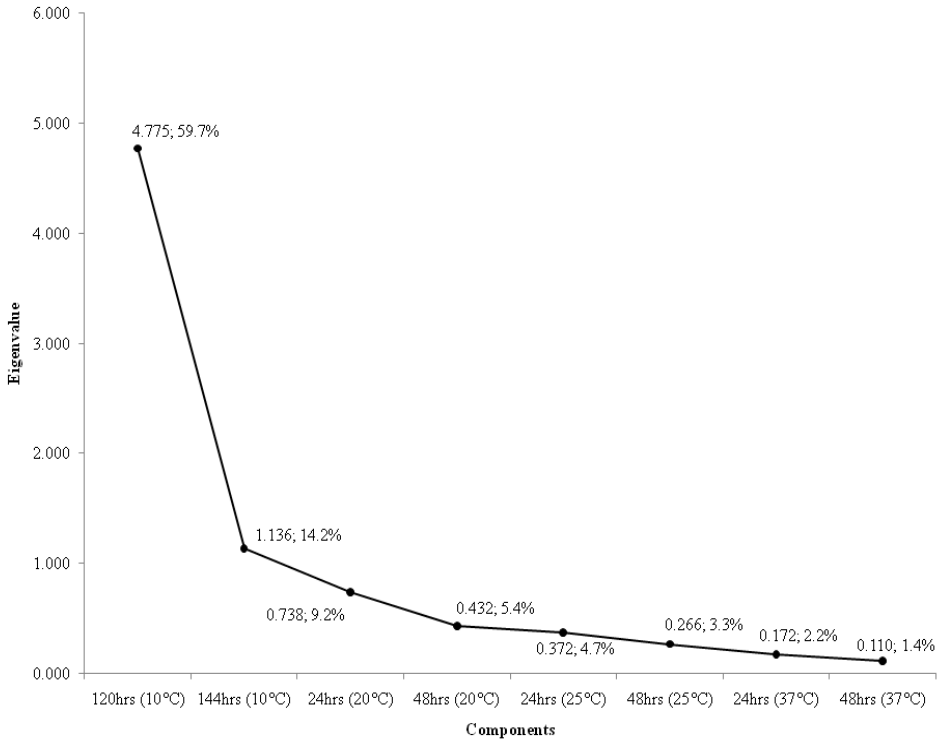


Figure 3.4. A scree plot based on eigenvalues obtained from principle component analysis of the temperature treatments. Data labels (left to right) are the eigenvalue followed by the proportion (%) of variation that the component contributes. Components 120 hours (10°C) and 144 hours (10°C) were distinct and accounted for most of the variation (59.7 and 14.2% respectively).

Table 3.2. Correlation matrix obtained from principle component analysis[a,b] of biofilm produced by *L. monocytogenes* strains under different temperatures at two different incubation times.

	120h (10°C)	144h (10°C)	24h (20°C)	48h (20°C)	24h (25°C)	48h (25°C)	24h (37°C)	48h (37°C)
120h (10°C)	**1**	0.6426	0.4658	0.4586	0.4614	0.4303	0.3272	0.4784
144h (10°C)	0.6426	**1**	0.3913	0.4289	0.3954	0.2771	0.1413	0.2571
24h (20°C)	0.4658	0.3913	**1**	0.8795	0.6693	0.7981	0.4041	0.6773
48h (20°C)	0.4586	0.4289	0.8795	**1**	0.6201	0.7675	0.3734	0.6991
24h (25°C)	0.4614	0.3954	0.6693	0.6201	**1**	0.7277	0.4946	0.6192
48h (25°C)	0.4303	0.2771	0.7981	0.7675	0.7277	**1**	0.5699	0.7185
24h (37°C)	0.3272	0.1413	0.4041	0.3734	0.4946	0.5699	**1**	0.4721
48h (37°C)	0.4784	0.2571	0.6773	0.6991	0.6192	0.7185	0.4721	**1**

a Shaded cells represent significant correlation at the 0.05 level (df = 92; $r \geq 0.264$[b]; $p<0.05$).
b Critical value for the correlation coefficient (r) obtained from Zar (1999a).

Univariate ANOVA indicated that biofilm production at different temperatures was significantly influenced by all of the qualitative characteristics of the *L. monocytogenes* isolates, with the exception of serotype and persistence status at 25°C (measurement time 1), origin, serotype and persistence status at 37°C (measurement time 1), and serotype at 37° for measurement time 2 (Table 3.3). Least significant difference (*t*-LSD) *post hoc* testing identified homogeneous subsets within the groups defined as significant by ANOVA (Table 3.4). Clinical isolates produced significantly greater biofilm growth than other isolates at higher temperatures, while environmental isolates produced significantly more biofilm at lower temperatures. Significant differences in biofilm production at low and high temperatures were also observed when the isolates were grouped by origin. Food production plant or human isolates produced significantly more biofilm at low and high temperatures respectively. The food production plant isolates (studied in Chapter 2) were observed to be distinct from most other isolates compared in this analysis. A scalar plot derived from the principle component analysis identified very high similarity between these strains when compared with those recovered from humans and other food production plant/product isolates (Figure 3.5).

When grouped by persistence status, sporadically recovered *L. monocytogenes* strains were found to produce significantly more biofilm than other isolates under all temperature treatments and measurement times with the exception of measurement time 1 at 25 and 37°C (Table 3.4). Similarly, when grouped by serotype, serotype 1/2a consistently produced significantly more biofilm than other serotypes at all temperatures except 37° C.

3.3.1.2 Microtitre plate biofilm production assay – effect of pH

Biofilm production by all of the *L. monocytogenes* strains tested in this study was affected by pH. High inter-strain variation and an increase in biofilm production between 24 and 48 hours incubation was noted for *L. monocytogenes* cultured in acidic pH conditions (pH 4.7 and pH 5.7; R^2 = 0.0108 and 0.0094 respectively). An increase in biofilm production between 24 and 48 hours incubation was also noted for *L. monocytogenes* when cultured in alkaline pH conditions (pH 8.5), and inter-strain variation remained high (R^2 = 0.3866). An increase in biofilm production by *L. monocytogenes* was also noted between 24 and 48 hours when cultured at pH 7.3, but variation between strains was low (R^2 = 0.8872).

Biofilm production was similar at both 24 and 48 hours incubation for the *L. monocytogenes* strains when cultured at pH 7.3. Increased biofilm production under acid and alkaline conditions was observed after 48 h incubation. In contrast, at 24 hours biofilm production under acidic and alkaline culture conditions was similar to, or less than, that produced at pH7.3 (Figure 3.6). Control strains FW04-0025 and ATCC 19114 produced very little biofilm after 24 hours of incubation at pH 7.3, and produced the least biofilm at 48 hours when compared with the factory strains.

Univariate ANOVA supported the observation that pH affected biofilm production by the *L. monocytogenes* isolates tested (Table 3.5). Significant differences were observed in biofilm production under all pH treatments and incubation times with the exception of the 24 hour measurements at pH 4.7 and 8.5. Least significant difference (*t*-LSD) *post hoc* testing defined the sporadic and environmentally persistent factory strains as a homogeneous group, significantly different from the control strains but not significantly different from each other (Table 3.6).

Table 3.3. Univariate ANOVA output[a] of biofilm formation under four incubation temperatures by *L. monocytogenes* isolates defined by qualitative characteristics.

Incubation Temperature	Incubation Time (H)	Qualitative Category			
		Nature	Origin	Persistence	Serotype
10°C	120	$F(2,92) = 4.46$, $p=0.01$	$F(7,87)=3.83$, $p<0.01$	$F(2,92)=4.69$, $p=0.01$	$F(7,87)=2.25$, $p=0.04$
	144	$F(2,92) = 8.78$, $p<0.01$	$F(7,87)=7.72$, $p<0.01$	$F(2,92)=19.21$, $p<0.01$	$F(7,87)=8.03$, $p<0.01$
20°C	24	$F(2,92) = 12.89$, $p<0.01$	$F(7,87)=6.93$, $p<0.01$	$F(2,92)=3.62$, $p=0.03$	$F(7,87)=3.90$, $p< 0.01$
	48	$F(2,92) = 14.31$, $p<0.01$	$F(7,87)=9.19$, $p<0.01$	$F(2,92)=6.80$, $p<0.01$	$F(7,87)=4.62$, $p<0.01$
25°C	24	$F(2,92) = 3.86$, $p=0.03$	$F(7,87)=2.96$, $p<0.01$	$F(2,92)=2.11$, $p=0.13$	$F(7,87)=1.53$, $p=0.17$
	48	$F(2,92) = 21.06$, $p<0.01$	$F(7,87)=9.22$, $p<0.01$	$F(2,92)=3.79$, $p=0.03$	$F(7,87)=4.20$, $p<0.01$
37°C	24	$F(2,92) = 3.58$, $p=0.03$	$F(7,87)=1.84$, $p=0.09$	$F(2,92)=2.39$, $p=0.10$	$F(7,87)=1.59$, $p=0.15$
	48	$F(2,92) = 17.58$, $p<0.01$	$F(7,87)=7.54$, $p<0.01$	$F(2,92)=3.30$, $p=0.04$	$F(7,87)=1.94$, $p=0.07$

a Values represent the *F* statistic (degrees of freedom) followed by the *p* value. Treatment categories that were not significant at $p = 0.05$ are shaded and were not considered for post hoc testing.

Table 3.4. Univariate ANOVA "post-hoc" output (*t*-test-least significant difference, *t*-LSD) of biofilm formation under four incubation temperatures by *L. monocytogenes* isolates defined by qualitative characteristics[a,b].

		Incubation Temperature							
		10°C		20°C		25°C		37°C	
	Qualitative Assignment	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2
Nature	Clinical (*n* = 53)	-1.82$_{a}$[0.167]	-1.72$_{a}$[0.171]	-1.40$_{a}$[0.195]	-1.18$_{a}$[0.239]	-1.49$_{a}$[0.269]	-0.92$_{a}$[0.264]	-0.94$_{a}$[0.292]	-0.69$_{a}$[0.223]
	Environmental (*n* = 22)	-1.79$_{a}$[0.167]	-1.42$_{b}$[0.171]	-1.56$_{a}$[0.195]	-1.36$_{a}$[0.239]	-1.75$_{a}$[0.269]	-1.42$_{b}$[0.264]	-1.26$_{b}$[0.292]	-1.03$_{b}$[0.223]
Origin	Food Factory (*n* = 15)	-1.63$_{a}$[0.425]	-1.21$_{a}$[0.407]	-1.38$_{a}$[0.491]	-1.07$_{a}$[0.574]	-1.58$_{b}$[0.698]	-1.95$_{a,b}$[0.669]	-1.17$_{a,b}$[0.783]	-0.85$_{a,b}$[0.573]
	Wallaby (*n* = 1)	-1.76$_{a,b}$[0.425]	-1.21$_{a}$[0.407]	-2.00$_{b}$[0.491]	-1.85$_{c}$[0.574]	-0.73$_{a}$[0.698]	-1.32$_{a,b,c}$[0.669]	-0.46$_{a}$[0.783]	-1.28$_{b,c}$[0.573]
	Human (*n* = 52)	-1.82$_{a,b}$[0.425]	-1.73$_{b}$[0.407]	-1.39$_{a}$[0.491]	-1.17$_{a,b}$[0.574]	-1.48$_{b}$[0.698]	-0.90$_{a}$[0.669]	-0.93$_{a}$[0.783]	-0.68$_{a}$[0.573]
	Bovine (*n* = 5)	-2.08$_{b}$[0.425]	-1.90$_{b}$[0.407]	-2.00$_{b}$[0.491]	-1.83$_{c}$[0.574]	-1.85$_{b}$[0.698]	-1.79$_{b,c}$[0.669]	-1.21$_{a,b}$[0.783]	-1.27$_{b,c}$[0.573]
	Food Factory / Product (*n* = 3)	-2.09$_{b}$[0.425]	-1.87$_{b}$[0.407]	-1.84$_{a,b}$[0.491]	-1.89$_{c}$[0.574]	-2.07$_{b}$[0.698]	-1.85$_{b,c}$[0.669]	-1.35$_{b}$[0.783]	-1.28$_{b,c}$[0.573]
	Ovine (*n* = 4)	-2.13$_{b}$[0.425]	-1.70$_{b}$[0.407]	-1.94$_{b}$[0.491]	-1.84$_{c}$[0.574]	-1.79$_{b}$[0.698]	-1.80$_{b,c}$[0.669]	-0.95$_{a,b}$[0.783]	-1.10$_{a,b,c}$[0.573]
	Food Product (*n* = 4)	-2.17$_{b}$[0.425]	-1.85$_{b}$[0.407]	-2.02$_{b}$[0.491]	-2.06$_{c}$[0.574]	-2.15$_{b}$[0.698]	-1.96$_{c}$[0.669]	-1.53$_{b}$[0.783]	-1.52$_{c}$[0.573]
Persistence	Persistent (*n* = 11)	-1.94$_{b}$[0.223]	-1.60$_{b}$[0.210]	-1.76$_{b}$[0.285]	-1.70$_{b}$[0.341]	-1.90$_{a}$[0.367]	-1.63$_{b}$[0.409]	-1.38$_{a}$[0.395]	-1.23$_{b}$[0.339]
	Sporadic (*n* = 10)	-1.59$_{a}$[0.223]	-1.15$_{a}$[0.210]	-1.31$_{a}$[0.285]	-0.95$_{a}$[0.341]	-1.55$_{a}$[0.367]	-1.14$_{a}$[0.409]	-1.33$_{a}$[0.395]	-0.79$_{a}$[0.339]
Serotype	1/2a (*n* = 43)	-1.88$_{a}$[0.422]	-1.71$_{a,b}$[0.381]	-1.44$_{a}$[0.505]	-1.27$_{a}$[0.610]	-1.48$_{a}$[0.692]	-0.95$_{a}$[0.720]	-0.87$_{a}$[0.746]	-0.77$_{a}$[0.638]
	1/2b (*n* = 5)	-1.96$_{a}$[0.422]	-1.52$_{a}$[0.381]	-1.99$_{c}$[0.504]	-1.89$_{d}$[0.610]	-1.76$_{a}$[0.692]	-1.67$_{c}$[0.720]	-1.16$_{a}$[0.746]	-1.31$_{b}$[0.638]
	1/2c (*n* = 2)	-2.05$_{b}$[0.422]	-1.97$_{b,c}$[0.381]	-1.88$_{c}$[0.504]	-1.94$_{d}$[0.610]	-2.05$_{a}$[0.692]	-1.70$_{c}$[0.720]	-1.26$_{a}$[0.746]	-1.48$_{c}$[0.638]
	3a (*n* = 8)	-1.82$_{a}$[0.422]	-1.75$_{a,b,c}$[0.381]	-1.47$_{a}$[0.504]	-1.39$_{b}$[0.610]	-1.50$_{a}$[0.692]	-1.06$_{b}$[0.720]	-1.14$_{a}$[0.746]	-0.84$_{a}$[0.638]
	4a (*n* = 2)	-1.96$_{a}$[0.422]	-1.65$_{a,b}$[0.381]	-2.06$_{c}$[0.504]	-1.71$_{c}$[0.610]	-1.76$_{a}$[0.692]	-1.76$_{c}$[0.720]	-1.09$_{a}$[0.746]	-1.10$_{b}$[0.638]
	4b (*n* = 17)	-1.97$_{a}$[0.422]	-1.83$_{a,b,c}$[0.381]	-1.72$_{a,b}$[0.504]	-1.59$_{c}$[0.610]	-1.85$_{a}$[0.692]	-1.58$_{b}$[0.720]	-1.29$_{a}$[0.746]	-1.07$_{b}$[0.638]
	4e (*n* = 2)	-2.15$_{b}$[0.422]	-2.11$_{c}$[0.381]	-1.66$_{a,b}$[0.504]	-1.72$_{c}$[0.610]	-1.97$_{a}$[0.692]	-1.60$_{b}$[0.720]	-1.20$_{a}$[0.746]	-0.95$_{b}$[0.638]

a. The values represent the $\log_{10}$ mean statistic for that group followed by the LSD in brackets. Significantly increased levels of biofilm production for each comparison are shaded. Homogeneous groups within each qualitative assignment were assigned the same subscript letter within the table. Values with a unique subscript letter differ significantly at $p = 0.05$.
b. *t*-Critical value for the nature, origin, persistence and serotype post-hoc comparisons was 1.986, 1.988, 1.986 and 1.988 respectively.

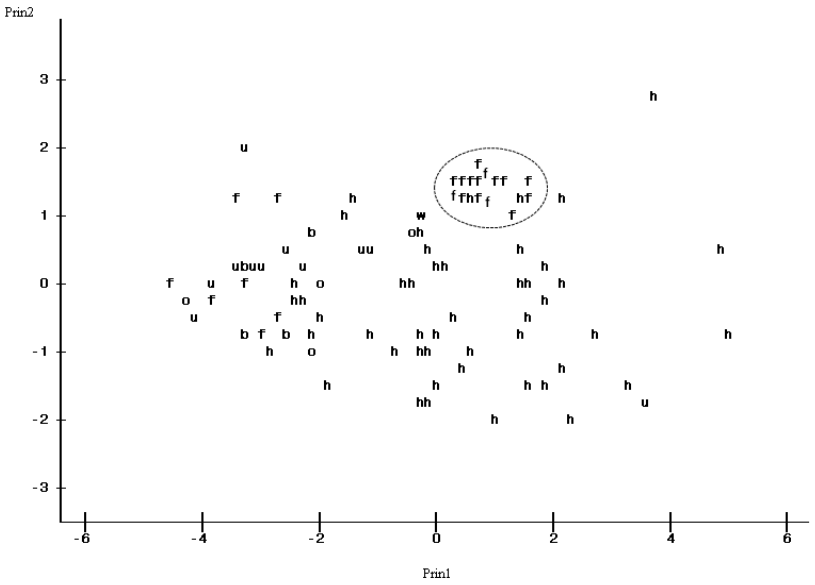


Figure 3.5. Principle component analysis scaling plot of the 95 *Listeria monocytogenes* strains based on a correlation matrix of association between incubation times and temperatures. The isolates have been grouped by the origins of each strain; human (h), food production plant /food production plant + product (f), ovine (O), bovine (b), wallaby (w) and unknown (u). Values represent standardised z-scores derived from the eigenvectors of the correlation matrix. Distances between objects are indicative of similarity in terms of the original variables; close = more similar. The food production isolates circled were obtained from a single food processing facility.

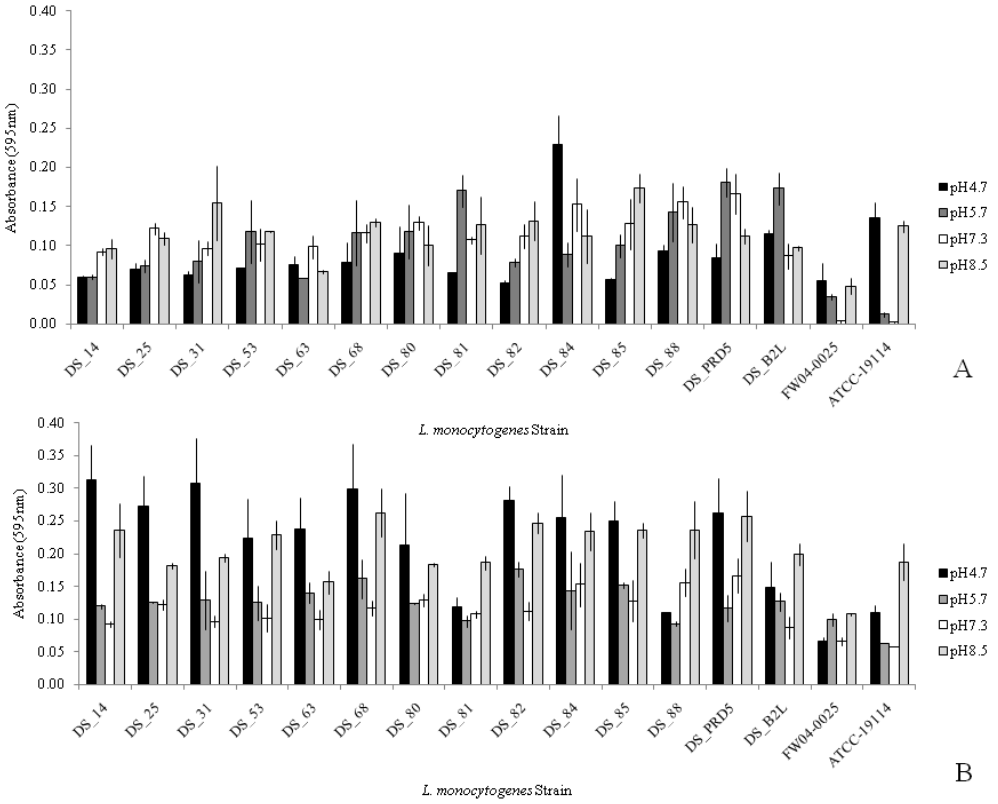


Figure 3.6. Biofilm production by *Listeria monocytogenes* strains following 24 hours (A) and 48 hours (B) incubation in BHI medium with pH adjusted to 4.7, 5.7, 7.3 and 8.5. Values represent the mean of three repeat absorbance measurements at 595nm. Error bars report one standard deviation from the mean of that measurement.

Table 3.5. Univariate ANOVA output[a] of biofilm formation in BHI media with the pH adjusted to 4.7, 5.7, 7.3 and 8.5 by *L. monocytogenes* isolates defined as environmentally persistent or sporadic (factory isolates), and the control strains FW04-0025 and ATCC 19114.

pH	Time	Output
4.7	24 h	$F(2,13) = 0.04$, $p = 0.96$
	48 h	$F(2,13) = 6.08$, $p = 0.01$
5.7	24 h	$F(2,13) = 7.66$, $p < 0.01$
	48 h	$F(2,13) = 3.98$, $p = 0.05$
7.3	24 h	$F(2,13) = 20.8$, $p < 0.01$
	48 h	$F(2,13) = 5.16$, $p = 0.02$
8.5	24 h	$F(2,13) = 1.02$, $p = 0.39$
	48 h	$F(2,13) = 3.42$, $p = 0.04$

a. Values represent the *F* statistic (degrees of freedom) followed by the *p* value. Outputs that were significant at $p = 0.05$ are shaded. Outputs found not to be significant were not considered for post hoc testing.

Table 3.6. Univariate ANOVA "post-hoc" output (*t*-test-least significant difference, *t*-LSD) of biofilm formation in BHI media with pH adjusted to 4.7, 5.7, 7.3 and 8.5 by *L. monocytogenes* isolates defined as environmentally persistent and sporadic (factory isolates), and the control strains FW04-0025 and ATCC 19114[a,b].

pH	Time	Persistent ($n = 4$)	Sporadic ($n = 10$)	Control ($n = 2$)
4.7	24 h	∞	∞	∞
	48 h	0.195_a[0.101]	0.251_a[0.101]	0.089_b[0.101]
5.7	24 h	0.144_a[0.058]	0.098_a[0.077]	0.024_b[0.077]
	48 h	0.128_a[0.039]	0.132_a[0.039]	0.081_b[0.039]
7.3	24 h	0.110_a[0.040]	0.123_a[0.040]	0.004_b[0.040]
	48 h	0.110_a[0.040]	0.123_a[0.040]	0.062_b[0.040]
8.5	24 h	∞	∞	∞
	48 h	0.208_a[0.059]	0.221_a[0.059]	0.148_b[0.059]

a. Homogeneous groups within each qualitative assignment are assigned the same subscript letter. Values with a unique subscript letter differ significantly at $p = 0.05$. The values represent the mean statistic for that group followed by the LSD in brackets. Significantly increased levels of biofilm production for each comparison are shaded. Cells marked ∞ correspond to insignificant treatment comparisons as determined by Univariate ANOVA and were not assessed post hoc.
b. Critical value for *t* was 2.160

3.3.2 Electron microscopy

Increased surface attachment was observed for both *L. monocytogenes* strains assessed (DS_14 and DS_81) under acidic and alkaline pH stress when compared to culture at pH 7.3. Surface attachment appeared greatest under alkaline growth conditions. No difference in surface attachment was observed between sporadic and the environmentally persistent *L. monocytogenes* strains (Figure 3.7).

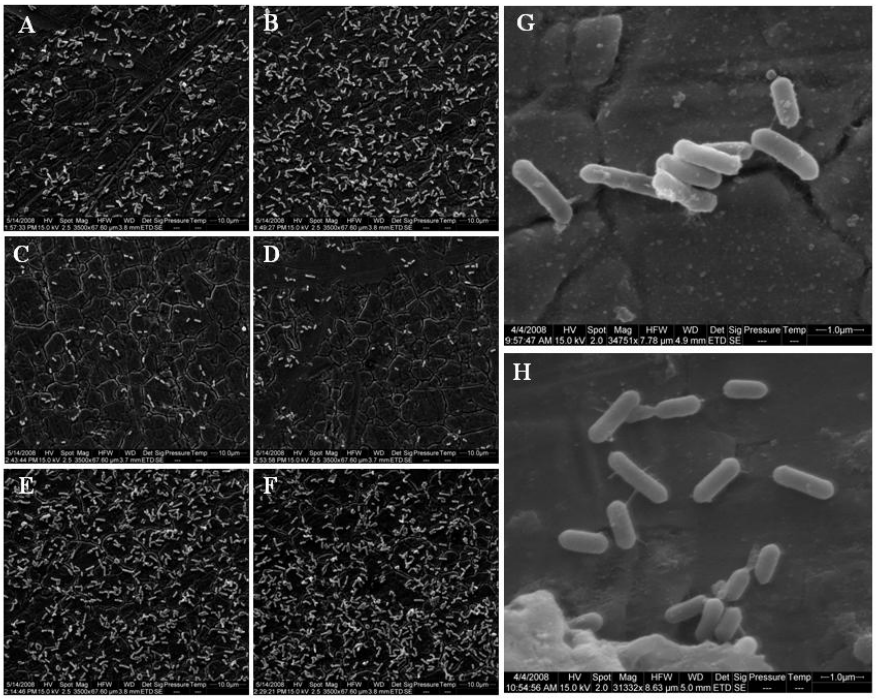


Figure 3.7 Electron micrograph of attachment by *L. monocytogenes* strains DS_14 (sporadic) and DS_81 persistent) under acid stress (pH 4.7), alkali stress (pH 8.5) and pH 7.3 conditions after 72 hours incubation at 25°C. Figures A and B show substratum attachment by strains DS_14 and DS_81 respectively under acid stress (3500 × Magnification). Figures C and D show attachment by strains DS_14 and DS_81 (respectively) under pH 7.3 conditions (3500 × Magnification). Figures E and F show attachment by strains DS_14 and DS_81 (respectively) under alkali stress (3500 × Magnification). Figures G and H show strains DS_81 and DS_14 (respectively) under higher magnification (31332 ×) attached to the stainless steel coupons under acidic (Figure G) and alkali (Figure H) pH stress.

3.4 Discussion

Many reports have demonstrated that environmental stresses influence biofilm production by *L. monocytogenes* (Harvey *et al.*, 2007; Moretro and Langsrud, 2004; Smoot and Pierson, 1998a, b). This has largely been attributed to environmentally induced alteration of the physiological characteristics of *L. monocytogenes* which, in turn, alters the ability of the organism to attach to substrata (Briandet *et al.*, 1999a, b; Giovannaci *et al.*, 2000; Tresse *et al.*, 2009). In addition, the chemical and physical parameters of the substrata and composition of the liquid medium has an influence, and, in combination with physiological adjustment by the bacterium, may dictate the rate, level and structure of biofilm assembly (Cuncliffe *et al.*, 1999; Tresse *et al.*, 2009). The present work supports those observations, with increased biofilm production being correlated with increasing temperature and acidic, or alkaline, growth conditions.

Production of biofilm is known to increase as incubation time increases, with progression through to a mature biofilm community (Davey and O'Toole, 2000; *see* Figure 3.1). At ambient temperatures, the initial stages of biofilm development in *L. monocytogenes* can be rapid, with adhesion observed within 20 minutes, deposition of matrix within 1 hour, stratification within 24 hours, and a mature biofilm community formed by 48 hours (Chae and Schraft, 2000; Mafu *et al.*, 1990; Wirtanen and Mattila – Sandholm, 1993). This pattern was observed in the current study with some differences noted depending on the incubation temperature. At lower incubation temperatures (10 and 20°C), initial establishment followed by formation of a mature biofilm (measurement times 1 and 2, respectively in this study) being correlated well with each other, and with biofilm production at these temperatures being moderate to low. However, at higher temperatures, biofilm production significantly increased, and variation between measurements increased. This was noted for the initial biofilm establishment (time 1) and formation of a mature biofilm (time 2) for the 37°C treatment, and formation of a mature biofilm (measurement time 2) for the 25°C treatment. Although temperature influences the growth /metabolic rate, the growth behaviour of *L. monocytogenes* in biofilms has been shown to differ from planktonic growth (Chae and Schraft, 2000). As such, increased growth rate alone cannot account for the observed increase in biofilm production at elevated temperatures.

The observed results may be attributed to physiological adjustment by the *L. monocytogenes* strains at elevated temperatures. More specifically, the observed increase in biofilm production at elevated temperatures may be the result of increased adhesion due to repressed expression of flagellin coupled with an increased cell surface hydrophobicity. At elevated temperatures, flagellin expression is down-regulated in *L. monocytogenes*, with complete repression reported at 37°C, inhibiting motility and inducing or contributing to a sessile state (Peel *et al.*, 1988; Way *et al.*, 2004). Additionally, flagellin is reported to be repressed during biofilm growth by *L. monocytogenes*, representing an essential component of the biofilm developmental process (Trémoulet *et al.*, 2002). However, flagellum-mediated attachment to substrata is a demonstrated means of early biofilm development (Lemon *et al.*, 2007). In the present study, significant biofilm development was observed at 37°C, despite reports that flagellum is often absent at these temperatures (Way *et al.*, 2004). These findings may be explained by the minimal movement of the culture broths in the present study minimising the need for motility to counteract shear and other forces

for adhesion, and suggests that other means of biofilm establishment (other than flagella) are available. This possibility is supported by Tresse *et al.* (2009) who found that *L. monocytogenes* can adhere to inert surfaces through a residual process independent of flagella, and several reports (Briandet *et al.,* 1999b; Chavant *et al.,* 2002; Smoot and Pierson, 1998a,b) demonstrate that the cell surface hydrophobicity of *L. monocytogenes* increases with temperature. On this basis, the combination of reduced motility, residual adhesion processes, and augmented adhesion to the hydrophilic PVC used in the current study would facilitate the formation of a biofilm at elevated temperatures. It is possible that this combination may actually supersede flagella-mediated attachment and initiation of biofilm development given the right conditions, resulting in comparably increased biofilm production.

Amongst the *L. monocytogenes* isolates tested, a subset of strains was identified that produced significantly more biofilm at elevated temperatures, as well as a subset of strains that significantly increased biofilm production at low temperatures. Strains that produced significantly more biofilm at higher temperatures were predominantly of clinical origin. Similarly, of the strains studied that produced significantly more biofilm at low temperatures, most were isolated from a single food processing facility (the DS_*n* strains). Interestingly, when grouped by serotype, *L. monocytogenes* strains corresponding to serotype 1/2a produced significantly more biofilm, irrespective of temperature, than other serotypes. Enhanced biofilm production by this serogroup has been reported previously, and is cause for concern given the known association with food products and clinical potential of these strains (Borucki *et al.,* 2003; Harvey *et al.,* 2007).

Increased biofilm production by clinical strains at elevated temperatures may be caused by physiological adjustment of the cells. However, these observations contradict those of previous studies, in which clinical strains rarely formed a biofilm (Kalmokoff *et al.,* 2001, Harvey *et al.,* 2007). The results imply that there may be a link between environmental survival (biofilm state) and pathogenesis (virulent state) in *L. monocytogenes*. As noted, flagellin is often repressed in *L. monocytogenes* at elevated temperatures, and could indirectly contribute to biofilm formation by induction of a sessile state. Flagella contribute to virulence in *L. monocytogenes* in the two-stage model introduced by O'Toole (2004), which suggests that bacteria can switch between a free-living virulent state, and a surface attached, less virulent state, depending on the environmental conditions. However, flagellin is now known not to

be essential for virulence in *L. monocytogenes*, forming only one part of the virulence induction mechanism (Way *et al.*, 2004).

Induction of virulence genes and, subsequently, virulence, in *L. monocytogenes* is initially controlled by a transcriptional activator known as Positive Regulatory Factor A (PrfA) (Chakraborty *et al.*, 1992). This, in turn, is regulated by three promoter regions; P*prfA*$_{p1}$, P*prfA*$_{p2}$ and P*plcA*. The first of these (P*prfA*$_{p1}$) is thermosensitive, inhibiting transcription of *PrfA* at low temperatures, while the second, P*prfA*$_{p2}$, is not (Johansson *et al.*, 2002). The third, P*plcA*, is autoregulated (Camilli *et al.*, 1993). Both P*prfA*$_{p1}$ and P*prfA*$_{p2}$ direct production of monocistronic *prfA* transcripts, while P*plcA* directs the transcription of both mono and bicistronic transcripts, with the bicistronic transcript requiring activation of PrfA (Johansson *et al.*, 2002; Gray *et al.*, 2006). Production of monocistronic *prfA* transcripts is not thermoregulated, and, as such, large numbers accumulate which, upon exposure to elevated temperatures such as those found within a host, result in rapid activation of *PrfA* transcription through P*prfA*$_{p1}$ (Gray *et al.*, 2006). Finally, thermosensitive P*prfA*$_{p1}$ appears to be directed by a σ^A homologue, associated with non-stressful conditions (Gruber and Gross, 2003; Nadon *et al.*, 2002). In contrast, P*prfA*$_{p2}$ is directed by σ^B, and associated with stress conditions. Furthermore, σ^B induction of P*prfA*$_{p2}$ is capable of inducing PrfA-mediated virulence at low temperatures (Cheng *et al.*, 2003; Gray *et al.*, 2006). As such, a range of virulence gene products may be expressed or repressed in *L. monocytogenes*, depending on the temperature or environmental stresses present, including secreted and cell surface associated proteins (Sokolovic *et al.*, 1993).

With this in mind, virulent *L. monocytogenes* strains may, at elevated temperatures, have made physiological adjustments conducive to surface attachment and production of biofilm. As such, in the absence of a host at elevated temperatures, and given appropriate environmental cues, clinical (virulent) *L. monocytogenes* strains may be successful biofilm producers by default. This hypothesis is supported by Kagkli *et al.* (2009), who determined that survival and persistence in *L. monocytogenes* has a dependence on temperature. Additionally, Roberts *et al.* (2009) determined that association between epidemic strains and listeriosis outbreaks may involve characteristics other than virulence manifestation, and suggested survival and growth in food-associated environments as a likely contributor. Furthermore, it has been demonstrated that, at 37°C, the invasion efficiency of some *L. monocytogenes* strains is significantly reduced (Roberts *et al.*, 2009; Kim *et al.*,

2004). On this basis it appears that, at elevated temperatures, there is overlap between the survival response and pathogenesis, with both states being innately conducive to biofilm initiation by a wide range of *L. monocytogenes* strains. Whether a particular strain shifts towards biofilm production or virulence may depend on both environmental signals and the intrinsic traits of that particular strain. As such, a divide may exist between *L. monocytogenes* strains that have enhanced survival and persistence strategies, and those with a greater propensity to cause disease. Such a distinction has been reported previously (Gray *et al.*, 2004), and is conducive to environmentally influenced, strain specific differences in the ability of *L. monocytogenes* to infect humans, persist within given environments and be dispersed in the food supply chain.

At low temperatures in the absence of other stresses, flagellum production and motility may increase in certain *L. monocytogenes* strains (Leifson and Palen, 1955). As such, low temperature conditions could favour flagella-mediated attachment, initiation of biofilm development and may, partly, explain the significantly increased biofilm production observed at low temperatures for the factory subset of *L. monocytogenes* strains (the DS_*n* strains). At the lowest incubation temperature, biofilm production was significantly different to the 37°C treatment ($r = 0.141$ (Time 1), 0.257 (Time 2); critical r value (df = 92) ≥ 0.264, $p<0.01$). It is plausible that this may reflect differences in the mode of biofilm formation, that is, there is a low temperature biofilm initiation mechanism (flagella-mediated attachment and biofilm initiation), and a high temperature biofilm initiation mechanism (induction of virulence, reduced motility, residual adhesion processes, and hydrophobic cell surface). However this was not observed for all of the isolates tested in this study (only the DS_*n* strains, all sourced from one food factory) suggesting that other factors influence the mode of biofilm production at low temperatures.

The DS_*n* strains used in this study were recovered from one food processing facility over a 12-month period, and have been resolved into five distinct molecular types by multilocus sequence typing (*see* Chapter 2). As such, the group represents a collection of non-clonal *L. monocytogenes* isolates that produce biofilm at a similar level under identical conditions. It seems feasible that the environmental conditions within the food processing facility may have been conducive to induction of a similar biofilm production response, and implies that a common set of environmental

conditions has selected for a group of unrelated *L. monocytogenes* strains with homogeneous biofilm producing responses.

Both environmentally persistent and sporadic strains were represented within the *L. monocytogenes* isolates tested in this study. When grouped by these qualitative traits, sporadic strains produced significantly more biofilm at all temperatures studied. This included the group of factory strains (with persistent and sporadic representatives) that dominated biofilm production at low temperatures, and differs from the findings of others that persistent strains consistently produced more biofilm (Borucki *et al.*, 2003; Lunden *et al.*, 2000). These results, and other work investigating biofilm production by *L. monocytogenes* (Harvey *et al.*, 2007; Moretro and Langsrud, 2004), suggest that persistence status alone may not be an indicator of the level of biofilm production (Harvey *et al.*, 2007; Moretro and Langsrud, 2004). While environmental persistence by *L. monocytogenes* strains is certainly augmented by enhanced biofilm production, evidence suggests that multiple persistent phenotypes exist, with variable biofilm production that may be dictated by the virulence status and environmental conditions to which they are habituated (Monk *et al.*, 2004; Moretro and Langsrud, 2004).

Exposure of the factory (DS_*n*) strains to pH stress supported this hypothesis. Independent of environmental persistence status, those strains homogeneously produced significantly more biofilm under conditions of alkaline and acidic stress after 48 hours when compared to the control strains. Additionally, initial biofilm establishment (measurement time 1) was not significantly different for the extremes of pH tested in this study (pH 4.7 and 8.5), suggesting that development of the mature biofilm is influenced more by moderately acidic and alkaline growth conditions than is attachment and initial establishment. This observation was supported by scanning electron microscopy of substratum attachment under identical conditions which also suggested that substratum attachment was greatest under alkaline conditions.

Biofilm production in response to pH stress may reflect environmental conditioning of the *L. monocytogenes* factory isolates. The inconsistency of the biofilm production response under these conditions suggests that the response may, to some extent, represent an innate response, induced by environmental preconditioning. This view is supported by Begley *et al.* (2009), who demonstrated that biofilm production by *L. monocytogenes* was similarly enhanced in the presence of bile. They propose that this may be a natural survival response facilitating

colonisation of the human gastrointestinal tract. This supports the notion that *L. monocytogenes* are universally capable of producing an innate biofilm production response, induced by the prevailing environmental conditions.

Kastbjerg *et al.* (2010) observed that when *L. monocytogenes* strains were exposed to sublethal concentrations of disinfectants, virulence gene expression was influenced. Exposure to sublethal concentrations of peroxy/chlorine compounds repressed virulence gene expression, while exposure to sublethal concentrations of quaternary ammonium compounds increased virulence gene expression. Resistance to these compounds by *L. monocytogenes* in a biofilm state has been described (Pan *et al.*, 2006). Again, this supports the hypothesis that there is overlap between the survival response and induction of virulence, with both states being innately conducive to biofilm initiation by a wide range of *L. monocytogenes* strains. Based on results from this study and others, an association between a saprophytic existence, biofilm formation and pathogenesis is suggested.

Based on the biofilm production responses to temperature and pH stress described here, and previously reported findings (Begley *et al.*, 2009; Kastbjerg *et al.*, 2010; Pan *et al.*, 2006; Monk *et al.*, 2004; Moretro and Langsrud, 2004), it is possible that the homogeneous biofilm production resulted from universal initiators. For example, the acidic or alkaline growth conditions may initiate a sequence of innate survival responses, similar to the observed temperature response and associated with virulence, that shift the cells into a biofilm production state. A conceptual model, summarising this hypothesis, is shown in Figure 3.8.

The factory from which the DS_*n* strains were recovered is routinely cleaned using an alkaline agent (Sumner, pers. comm., 2010). *L. monocytogenes* strains capable of surviving routine exposure to acidic or alkaline cleansing and sanitising agents, and common environmental conditions, may be selectively enriched, and capable of rapidly undergoing this innate physiological shift under those specific conditions.

In conclusion, in this study it has been demonstrated that biofilm production in *L. monocytogenes* has both intrinsic and extrinsic elements. A temperature-specific biofilm production response was identified, and environmentally-induced homogeneous biofilm production by non-clonal *L. monocytogenes* strains was highlighted. The work supports the notion that distinct and environmentally inducible mechanisms of biofilm initiation exist, and that there may be an overlap between these mechanisms and pathogenesis mechanisms in *L. monocytogenes*. Finally, the

study showed that biofilm production is not necessarily a determinant of environmental persistence, suggesting that other factors may define the persistent *L. monocytogenes* phenotype. These findings suggest an association between *L. monocytogenes* survival strategy and virulence. This has considerable food safety implications. More work is required to fully understand the association and to identify the specific elements that are involved. This information would help guide preventative measures, augment current cleansing and sanitising regimes, and aid in the development of targeted controls against high-risk *L. monocytogenes* populations.

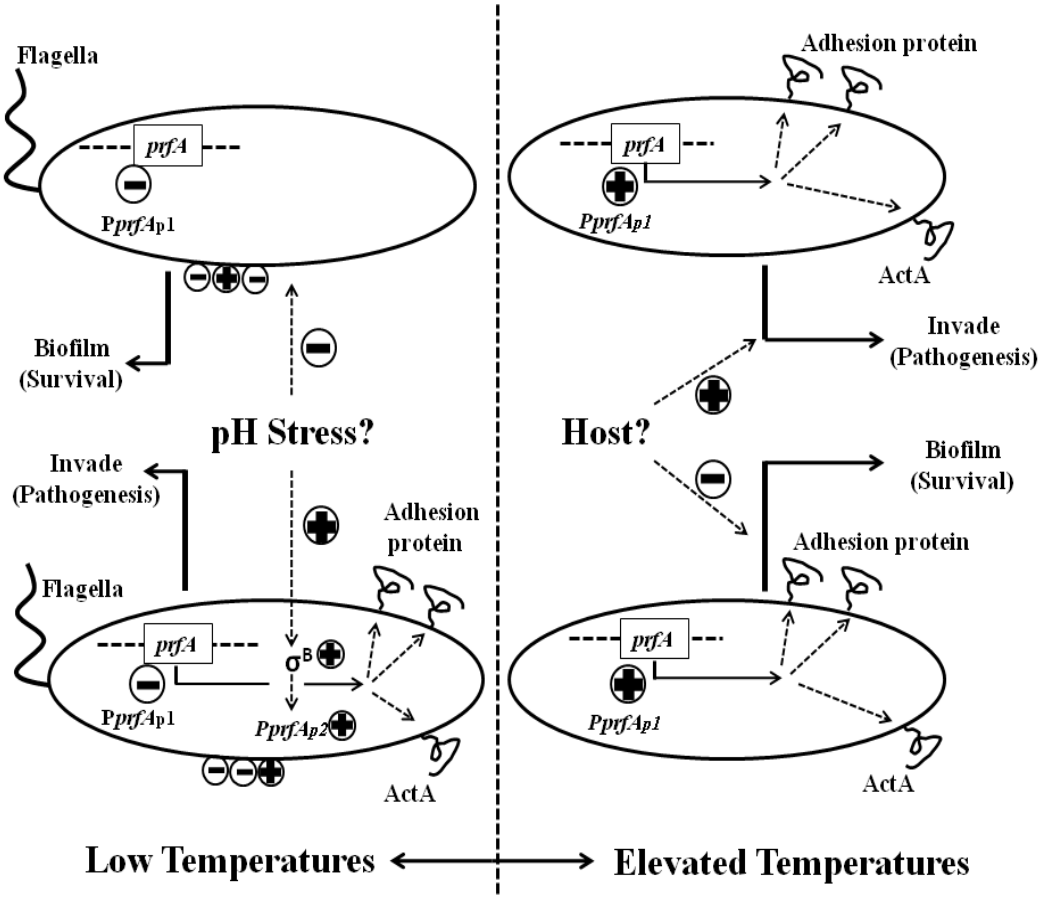


Figure 3.8. A descriptive model of the proposed association between temperature, environmental stress (pH), virulence and biofilm production in *L. monocytogenes*. At high temperatures, such as those found in a mammalian or avian host, a virulent state is initiated by P*prfA*$_{p1}$ directed induction of *prfA* transcription, likely controlled by σ^A (Gray *et al.*, 2006). This results in the production of virulence proteins including known adhesion elements such as actin filaments (ActA) (Sokolovic *et al.*, 1993). Additionally, cell surface hydrophobicity increases, enhancing adhesion to certain substrata such as PVC. In the absence of a host, many of the physiological attributes induced through transformation to a virulent state are conducive to the formation of a biofilm, and may represent a survival "shunt". In non-stressful environments at low temperatures, PrfA mediated induction of virulence is inhibited. Cell surface hydrophobicity is decreased, and in combination with expression of flagella, allows the cell to overcome the repulsive forces of a wide range of substrata, facilitating attachment and biofilm formation. Under conditions of acid or alkaline stress at low temperatures, σ^B can induce expression of PrfA and associated virulence proteins under the direction of P*prfA*$_{p2}$, augmenting the biofilm production (survival) response.

CHAPTER 4
MUDPIT BASED PROTEOMIC COMPARISON OF ALKALINE ADAPTED, ENVIRONMENTALLY PERSISTENT *LISTERIA MONOCYTOGENES* STRAINS

4.1 Introduction

Persistent contamination of food factory environments by *Listeria monocytogenes* is well documented (Holah *et al.*, 2004; Lunden *et al.*, 2003; Lunden *et al.*, 2002; Lunden *et al.*, 2000; Moretro and Langsrud, 2004; Pan *et al.*, 2006). However, *L. monocytogenes* physiological mechanisms that facilitate the persistent contamination remain to be definitively characterised. Confounding this pursuit is the observation that different strains of *L. monocytogenes* vary in their ability to persist within the environment (Holah *et al.*, 2004; Lunden *et al.*, 2003; Lunden *et al.*, 2000; Moretro and Langsrud, 2004). *L. monocytogenes* mechanisms of environmental persistence are thought to involve differential tolerance of physiological stresses (Gandhi and Chikindas, 2007; Gray *et al.*, 2006; Heavin *et al.*, 2009; Moretro and Langsrud, 2004; Kastbjerg *et al.*, 2009). Examples of these stresses include temperature extremes, osmotic pressures and pH stress, and there is evidence suggesting that exposure to physiological stress may increase the infective potential of *L. monocytogenes* (Kim *et al.*, 2006; Anderson *et al.*, 2007; Gray *et al.*, 2006; O'Driscoll *et al.*, 1996). Consequently, factors contributing to environmental persistence may increase the potential for transmission of serious food-borne disease.

In terms of stress tolerance in *L. monocytogenes*, the pH response has received considerable attention due to the resistance the organism demonstrates and the routine use of this hurdle within food processing environments (Kastbjerg *et al.*, 2009; Giotis *et al.*, 2007b; O'Driscoll *et al.*, 1996; Pan *et al.*, 2006; Wiedmann *et al.*,1998). Food processing factories often employ a combination of alkaline and acid cleansers and sanitisers as part of a chemically active cleansing and sanitising regime. Examples include moderately alkaline disinfectants such as tri-sodium phosphates and ammonium salts of phosphate, highly alkaline disinfectants such as caustic soda and caustic potash, and both organic and inorganic acid disinfectants such as hydroxy-acetic acid and hydrochloric acid respectively. The effectiveness of these regimes depends on a number of variables including characteristics of the application surface, exposure times, temperature, concentration of the agent and a number of

other chemical and biological factors (Somers and Wong, 2004). Food processing factories are complex environments. All cleansing and sanitising operations are challenged by temporal and physical elements that may detrimentally affect these regimes. Should any of the factors that can reduce sanitation efficiency fail to be adequately addressed, cleanser/sanitiser efficiency may be reduced or even eliminated completely. In such situations, microbial populations may be subjected to fluxes in cleanser / sanitiser concentrations, possibly enabling microbial acclimatisation and survival in otherwise lethal pH conditions (Wiedmann *et al.,* 1998; Kastbjerg *et al.,* 2009).

Recent work investigating the pH stress response in *L. monocytogenes* has predominantly focused on low pH environments. It is now accepted that under mildly acidic conditions, an acid tolerance response may be induced, which can then afford protection against more severe acid exposures (Davis *et al.,* 1996; Heavin *et al.,* 2009; Gahan *et al.,* 1996; O'Driscoll *et al.,* 1996). Knowledge of the molecular mechanisms that underpin this response is increasing, with expression of specific protective and supportive factors identified as an important component. These include transcriptional regulators, ATP synthase and the molecular chaperone GroEL, F_0F_1- ATPase-mediated maintenance of intracellular pH, the glutamate decarboxylase system, induction of stress response genes by a two-component regulatory system and the general stress response regulator σ^B (Abram *et al.,* 2008a; Wiedemann *et al.,* 1998; Heavin *et al.,* 2009; Kazmierczak *et al.,* 2003; Sokolovic *et al.,* 1993).

In contrast to the acid response, very little is understood of the alkaline adaptation/stress response in *L. monocytogenes*. Much of what is known is derived through comparisons with alkaliphilic microbes and the relatively small amount of scientific literature available on the topic (Giothis *et al.,* 2008; Padan *et al.,* 2005). Given the role alkaline agents play in the cleaning and sanitation regimes of many food processing environments, knowledge of the molecular mechanisms driving alkaline tolerance in *L. monocytogenes* is essential to gain a more thorough understanding of pH adaptation in this species.

The majority of work investigating the alkali stress response of *L. monocytogenes* is based on the organism's response to pH shock and the cross protective effects of alkali adaptation. These have demonstrated that the alkali tolerance response (AlTR), and, therefore, the alkali shock and cross protective responses, is dependent on *de novo* protein synthesis (Giotis *et al.,* 2008). The study

described in this chapter investigated the protein expression of persistent and sporadic *L. monocytogenes* strains acclimatised to alkaline growth conditions. The analysis used two-dimensional liquid chromatography coupled with electrospray ionisation tandem mass spectrometry, commonly termed multidimensional protein identification technology, or MuDPIT (Delahunty and Yates, 2005). Soluble protein expression profiles of cell fractions from *L. monocytogenes* strains in planktonic mid-exponential and stationary growth states at pH7.3 and adapted to pH8.5 were compared. This was intended to identify differences in the alkaline adaptation response of persistent verses sporadic *L. monocytogenes* factory contaminants, with the hope of elucidating the proteins aiding persistence within factory environments.

4.2 Materials and Methods.

4.2.1 Bacterial strains and culture conditions

Environmentally persistent and sporadic *L. monocytogenes* strains were studied (Table 4.1). Bacterial cultures were recovered from cold storage (Appendix 1.1) and grown in 10 mL of Brain – Heart Infusion broth (CM225, 'BHI'; OXOID, Australia; Appendix 2.2) incubated at 25°C for 20 h. The medium was adjusted to pH8.5 and pH7.3 (±0.1) through addition of measured volumes of 4 M NaOH (Sigma-Aldrich, Castle Hill, Australia) and HCl (Sigma-Aldrich, Castle Hill, Australia). After autoclaving, the pH of both media (pH7.3 and pH8.5) was determined using an Orion 250A pH meter (Orion Research Inc, USA), and further adjusted using sterile NaOH and HCl if required. The initial culture conditions were repeated over a period of 7 days by transferring (daily) a 100 µL aliquot of the cultures to 9.9 mL of fresh BHI to acclimatise the cultures to the growth conditions. Finally, two replicate (2 × biological replicate) 10 mL cultures at each pH condition were prepared and grown to early stationary phase ($OD_{600} \approx 0.9 \pm 0.1$) and mid – exponential phase ($OD_{600} \approx 0.4 \pm 0.1$) at 25°C.

Table 4.1 *Listeria monocytogenes* strains used in this study.

Isolate	Source	Details
DS_14[a]	Australian food production factory (smallgoods)	Recovered (once only) from the inner floor surface of a hopper (Chapter 2)
DS_81[b]	Australian food production factory (smallgoods)	Recovered twice over a 12 month period from the switch box of a frankfurter deskinner (Chapter 2)
102-195-242-S-1[c]	United Kingdom food production factories (ready-to-eat foods)	Recovered from 44 environmental and 37 product samples over a period of 21 months.

a) Isolate DS_14 was recovered once from the same Australian food processing plant sampling, and was considered a sporadic contaminant.
b) Isolate DS_81 is an environmentally persistent *L. monocytogenes* strain obtained from an Australian food processing plant twice over a period of 12 months.
c) Isolate 102-195-242-S-1 is a demonstrated environmentally persistent strain (Holah *et al.*, 2004) and was used as an environmentally persistent control strain.Kindly provided by Dr John Holah of the Campden and Chorleywood Food Research Association, United Kingdom (Holah *et al.*, 2004).

4.2.2 Preparation of bacterial extracts for MuDPIT analysis

The bacterial cultures were centrifuged at 14000 × g for 5 minutes at 4° C in an Eppendorf 51417 centrifuge and the supernatant was discarded. The cell pellets were gently resuspended (washed) in phosphate buffered saline (Appendix 1.6), transferred to 1.5 mL Eppendorf Protein LoBind microcentrifuge tubes (Sigma-Aldrich, Castle Hill, Australia), and centrifuged again at 14000 × g for 5 minutes. The wash was repeated three times. Following the final wash, the supernatant was discarded and the cell pellet frozen in liquid nitrogen. The pellet was thawed on ice for 15 minutes. A Qproteome bacterial protein preparation kit (Qiagen Pty Ltd, Victoria, Australia) was used to extract soluble proteins from the lysed cell pellets according to manufacturer instructions. Approximate concentration of the protein extracts was determined by standard Bradford assay (Sigma-Aldrich, Castle Hill, Australia) in a 96-well microplate format (Greiner Scientific, Australia) using a 2mg/mL Bovine Serum Albumin (BSA) standard (Sigma-Aldrich, Castle Hill, Australia). Absorbance at 595nm was measured using a Benchmark microplate reader (BioRad, USA).

A volume of protein extract containing ≈50 µg of protein was transferred to a clean Eppendorf Lobind microcentrifuge tube, frozen with liquid nitrogen, and freeze-dried using a vacuum freeze dryer (Dynavac, Australia) for approximately 5 hours. The concentrated sample was resuspended in 20 µL of 8 M urea, 100 mM ammonium bicarbonate buffer (Sigma-Aldrich, Castle Hill, Australia; Appendix 1.9) in water. The sample was reduced for 1 hour at room temperature using 5 µL of 50 mM dithiothreitol (Sigma-Aldrich, Castle Hill, Australia) in 100 mM of the

ammonium bicarbonate buffer. Following reduction, the sample was alkylated for one hour at room temperature using 5 µL of 200 mM iodoacetamide (Sigma-Aldrich, Castle Hill, Australia) in 100 mM ammonium bicarbonate buffer. Unreacted alkylation agent was consumed through addition of a further 20 µL of reducing solution (ammonium bicarbonate buffer) followed by one hour incubation at room temperature.

Prior to enzymatic digestion, the urea concentration was reduced through addition of 170 µL of digest solution (Appendix 1.10). Promega sequencing grade modified trypsin (Sigma-Aldrich, Castle Hill, Australia) suspended in digest solution was added to the sample to produce a 1:50 enzyme to substrate ratio. The sample was gently mixed and incubated at 37° C for approximately 12 hours. Following incubation, digestion was halted by adding 10 µL of 10% formic acid in water (Sigma-Aldrich, Castle Hill, Australia). The sample was centrifuged at 14000 × g for 5 minutes to remove insoluble material and the supernatant was transferred to a high pressure liquid chromatography vial (Waters, U.S.A.) for MuDPIT analysis.

The tryptic digests were analysed using MuDPIT. The method is based on coupled liquid chromatography/mass spectrometry and used a nanoflow triphasic MuDPIT system consisting of a C18 capillary trap followed by a strong cation exchange resin (SCX) stage and an analytical C18 nano-column. High pressure liquid chromatography buffers used in this analysis are outlined in Appendix 1.11.

4.2.3 MuDPIT analysis

Fifty microlitres of tryptic digest was loaded onto the C18 capillary trap at a flow rate of 35 µL/min. During sample loading the SCX and analytical columns were switched via a valve out of line of the C18 trap, with the trap being washed to waste to ensure salts and other non-peptide materials were not introduced into the mass spectrometer. After 10 minutes (sample loading) the SCX and analytical columns were switched in-line with the capillary trap and the flow reduced via a splitter to 250 nL/min. A five step MuDPIT gradient was performed as described in Appendix 1.12. Complete analysis time was 10.5 hours. To ensure contamination between runs did not occurr a 1.5 hour cleanup method was performed.

The mass spectrometry component used a Themoelectron LTQ Orbitrap (Thermo Fisher Scientific, Australia). This is a hybrid mass spectrometer, consisting of a 2 dimensional ion trap capable of low resolution MS/MS (LTQ part), and an

electrostatic trap capable of performing high resolution, high mass accuracy analysis (Orbitrap part). During the MuDPIT analysis the mass spectrometer was operated in "data dependant" tandem mass spectrometry (MS/MS) mode. A "survey" scan performed in the electrostatic Orbitrap identified the possible parent peptide masses with a precursor-ion mass tolerance of 10 ppm. The mass spectrometer then made a "data dependant" decision as to what peaks would be subjected to tandem mass spectrometry. The top 6 peaks were fragmented in the ion trap, producing MS/MS data. This was operated with a fragment-ion mass tolerance of 0.5 Daltons. The process took under 2 seconds to perform and was cycled continually throughout the MuDPIT run.

The proteins in the sample were identified by matching the tandem mass spectra data against the total *L. monocytogenes* database of the National Centre for Biotechnology Institute using the Computational Proteomics Analysis System (CPAS) Version 8.1, a web-based system built on the LabKey Server for managing, analysing, and sharing high volumes of tandem mass spectrometry data (www.labkey.org). Searches were semi-tryptic with fixed modification (cysteine carbamidomethylation - 57 Dalton) allowing no missed cleavages using the X!Tandem algorithm in the method described by Craig and Beavis (2004). Searches were run through the Trans Proteomic Pipeline (TPP; Version 3.4) for statistical purposes.

The TPP analysis utilised the "Peptide Prophet" and "Protein Prophet" algorithms as previously described (Keller *et al.*, 2002). In addition, a decoy (reversed) database search was performed to assess the likelihood of incorrect peptide – protein matches. An identification integration analysis was applied to both TPP outputs. This enabled the level of false positive peptide and protein identifications to be estimated and controlled. Identifications with a Peptide Prophet score of <0.5, protein group probabilities of <0.5, and protein identifications with a Protein Prophet score of <0.5 were not considered for further analysis. Protein identifications having single unique peptide assignments were included and assessed based on the percentage of spectrum identifications and percentage coverage of the identified protein. An overview of the MuDPIT workflow is presented in Figure 4.1. Data grouping, assembly and refinement were performed using Microsoft Excel® 2007 (Microsoft Inc., U.S.A.).

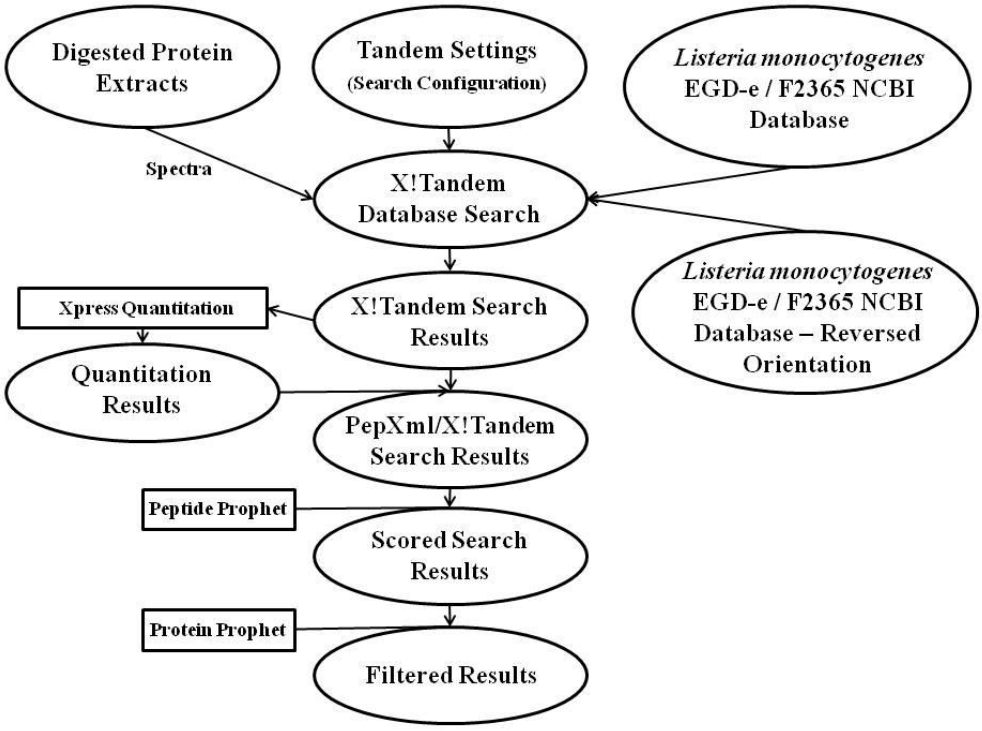


Figure 4.1. The MuDPIT work flow applied in the current study.

4.2.4 Functional grouping and biological role assignment

Functional grouping of protein identifications was performed manually using the J. Craig Venter Institute Comprehensive Microbial Resource (JCVI-CMR) (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntlm01) *L. monocytogenes* EDG-e primary annotation summary database. Biological role assignment of protein identifications was performed manually using the "Genolist" *L. monocytogenes* serovar 1/2a EGD-e database (Version 3) (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList.woa/wa/goToTaxoRank?level=Listeria%20monocytogenes%20EGD-e), and Kyoto Encyclopedia of Genes and Genomes (KEGG) *L. monocytogenes* serovar 1/2a EGD-e database (http://www.genome.jp/kegg/). Identifications corresponding to *L. monocytogenes* strains other than EGD-e were assigned a functional role manually based on proteins with similar function allocated within the JCVI-CMR, Genolist and KEGG, EGD-e databases. A key detailing the functional assignments and the codes (manually assigned) used throughout this study is detailed in Table 4.2.

Table 4.2 Major functional assignments, and their assigned codes, used in the present study. The functional assignments are based on the JCVI-CMR *L. monocytogenes* EGD-e functional ontology system.

Major Functional Category		Minor Functional Category	
Code	Functional Role	Code	Functional Role
A	Amino Acid Biosynthesis	1	Aromatic
A	Amino Acid Biosynthesis	2	Aspartate
A	Amino Acid Biosynthesis	3	Glutamate
A	Amino Acid Biosynthesis	4	Pyruvate
A	Amino Acid Biosynthesis	5	Serine
A	Amino Acid Biosynthesis	6	Histidine
B	Biosynthesis of Cofactors, Prosthetics, Carriers	7	Folate
B	Biosynthesis of Cofactors, Prosthetics, Carriers	8	Haeme, Porphoryn and Cobalamin
B	Biosynthesis of Cofactors, Prosthetics, Carriers	9	Lipoate
B	Biosynthesis of Cofactors, Prosthetics, Carriers	10	Menaquinone and Ubiquinone
B	Biosynthesis of Cofactors, Prosthetics, Carriers	11	Molybdopterin and Molybdenum
B	Biosynthesis of Cofactors, Prosthetics, Carriers	12	Panthotenate and CoA
B	Biosynthesis of Cofactors, Prosthetics, Carriers	13	Pyrodoxine
B	Biosynthesis of Cofactors, Prosthetics, Carriers	14	Riboflavin and Similar
B	Biosynthesis of Cofactors, Prosthetics, Carriers	15	Glutathione and Similar
B	Biosynthesis of Cofactors, Prosthetics, Carriers	16	Thiamine
B	Biosynthesis of Cofactors, Prosthetics, Carriers	17	Pyrimidine Nucleotides
B	Biosynthesis of Cofactors, Prosthetics, Carriers	18	Other
C	Cell Envelope	19	Surface Structures
C	Cell Envelope	20	Biosynthesis and Degradation of Murein and Peptidoglycan
C	Cell Envelope	21	Lipopolysaccharides
C	Cell Envelope	22	Other
D	Cellular Processes	23	Cell Division
D	Cellular Processes	24	Chemotaxis and Motility
D	Cellular Processes	25	Detoxification
D	Cellular Processes	26	Transformation
D	Cellular Processes	27	Toxin production and resistance
D	Cellular Processes	28	Pathogenesis
D	Cellular Processes	29	Adaptation to Atypical Conditions
D	Cellular Processes	30	Other
E	Central Intermediary Metabolism	31	Amino-Sugars
E	Central Intermediary Metabolism	32	Phosphate
E	Central Intermediary Metabolism	33	Polyamines
E	Central Intermediary Metabolism	34	Sulphur
E	Central Intermediary Metabolism	35	Nitrogen Metabolism
E	Central Intermediary Metabolism	36	Other
F	DNA Metabolism	37	Replication, Recombination and Repair
F	DNA Metabolism	38	Restriction and Modification
F	DNA Metabolism	39	Degradation of DNA
F	DNA Metabolism	40	Other
G	Disrupted Reading Frame	41	Disrupted Reading Frame
H	Energy Metabolism	42	Aerobic
H	Energy Metabolism	43	Amino Acids and Amines
H	Energy Metabolism	44	Anaerobic
H	Energy Metabolism	45	ATP Production, Proton Motive Force
H	Energy Metabolism	46	Electron Transport Chain
H	Energy Metabolism	47	Entner-Doudoroff
H	Energy Metabolism	48	Fermentation
H	Energy Metabolism	49	Glycolysis / Gluconeogenesis
H	Energy Metabolism	50	Pentose Phosphate Pathway

H	Energy Metabolism	51	Pyruvate Dehydrogenase
H	Energy Metabolism	52	Sugars
H	Energy Metabolism	53	TCA Cycle
H	Energy Metabolism	54	Methanogenesis
H	Energy Metabolism	55	Biosynthesis / Biodegradation of Polysaccharides
H	Energy Metabolism	56	Photosynthesis
H	Energy Metabolism	57	Other
I	Fatty Acid and Phospholipid Metabolism	58	Biosynthesis
I	Fatty Acid and Phospholipid Metabolism	59	Degradation
I	Fatty Acid and Phospholipid Metabolism	60	Other
K	Mobile and Extrachromosomal Elements	61	Plasmid
K	Mobile and Extrachromosomal Elements	62	Prophage
K	Mobile and Extrachromosomal Elements	63	Transposon
L	Protein Fate	64	Peptide Secretion and Trafficking
L	Protein Fate	65	Modification and Repair
L	Protein Fate	66	Folding and Stability
L	Protein Fate	67	Degradation of Proteins, Peptides and Glycoproteins
M	Protein Synthesis	68	tRNA Amino Acids
M	Protein Synthesis	69	Ribosome Synthesis and Modification
M	Protein Synthesis	70	tRNA-RNA Base Modification
M	Protein Synthesis	71	Translation Factors
M	Protein Synthesis	72	Other
N	Purines, Pyrimidines, Nucleosides, Nucleotides	73	2-Deoxy Metabolism
N	Purines, Pyrimidines, Nucleosides, Nucleotides	74	Nucleoside Interconversion
N	Purines, Pyrimidines, Nucleosides, Nucleotides	75	Purine Ribonucleotide Biosynthesis
N	Purines, Pyrimidines, Nucleosides, Nucleotides	76	Pyrimidine Ribonucleotide Biosynthesis
N	Purines, Pyrimidines, Nucleosides, Nucleotides	77	Salvage of Nucleotides
N	Purines, Pyrimidines, Nucleosides, Nucleotides	78	Other
O	Regulatory Functions	79	DNA Interactions
O	Regulatory Functions	80	RNA Interactions
O	Regulatory Functions	81	Protein Interactions
O	Regulatory Functions	82	Other
P	Signal Transduction	83	Phospho-Transferase System
Q	Transcription	84	Degradation of RNA
Q	Transcription	85	DNA Depolarising RNA polymerase
Q	Transcription	86	Transcription Factors
Q	Transcription	87	RNA Processing
Q	Transcription	88	Other
R	Transport and Binding	89	Amino Acids, Peptides and Amines
R	Transport and Binding	90	Anions
R	Transport and Binding	91	Carbohydrates, Alcohols and Acids
R	Transport and Binding	92	Cation, Iron
R	Transport and Binding	93	Nucleosides, Purines, Pyrimidines
R	Transport and Binding	94	Other
S	Unknown Function	95	Unknown Substrate
S	Unknown Function	96	Enzyme of Unknown Specificity
S	Unknown Function	97	General
S	Unknown Function	98	Viral Functions
S	Unknown Function	99	Unknown Substrate

4.2.5 Differential protein abundance

Relative protein abundances were determined by the spectral counting method (Wang *et al.*, 2003) using TPP Xpress quantitation software (Version 2.1) in conjunction with X!Tandem analysis. Spectral counts were first normalised to account for sampling depth as described by Beissbarth *et al.* (2004):

$$O_i = n_1 t_2 / t_1 + \lambda;\ O_j = n_2 + \lambda$$

Where O_i and O_j are the normalised spectral counts for samples 1(i) and 2(j) respectively, n_1 and n_2 are the raw spectral counts for samples 1($_1$) and 2($_2$) respectively, t_1 and t_2 are the sum of the spectral counts for samples 1($_1$) and 2($_2$) respectively, and λ is a pseudo-spectral count (0.5). After normalisation, a likelihood ratio test for independence (G-test) was performed according to the method described by Sokal and Rohlf (1995):

$$G = 2\sum_{ij} O_{ij} \cdot \ln(O_{ij}/E_{ij})$$

Where E_i and E_j are the expected spectral counts for samples 1(i) and 2(j) respectively, calculated as described by Sokal and Rohlf (1995):

$$E_{ij} = (O_i + O_j) / 2$$

To minimise risk of type I error, a William's correction was applied according to the method described by Sokal and Rohlf (1995), resulting in an adjusted G-statistic:

$$G_{adj} = G/w.$$

Where w is a Williams's correction, calculated as described by Sokal and Rohlf (1995):

$$w = 1 + \frac{\left(\frac{n}{n_1} + \frac{n}{n_2} - 1\right)\left(\frac{n}{x} + \frac{n}{y} - 1\right)}{6n}.$$

Where n is the combined total spectra count for the samples being compared, n_1 is the total spectra count for sample 1, n_2 is the total spectra count for sample 2, x is the total spectra count for the target protein and y is the total spectra count for any other protein.

Differential protein expression between strains and between treatments was assessed for significance by comparing the adjusted *G*-value for each protein against a *Chi*-square distribution table using one degree of freedom (Zar, 1999b). Based on this, *G*-values greater than 3.841 were considered significant (χ^2; $p<0.05$). Protein identifications present in both the persistent factory strain (DS_81) and the persistent control strain (102-195-242) with significantly different spectral abundances relative to the sporadic strain (DS_14) are presented.

4.3 Results

4.3.1 Functional distribution of proteins relative to *L. monocytogenes* strain, growth phase, and pH of the growth media

1110 unique proteins were identified, from all strains combined, following integration analysis (716 for strain 102-195-242, 801 for strain DS_81, and 748 for strain DS_14; Appendix 5.1). These were assigned a functional role, and the proportion of proteins corresponding to each functional role (% relative to the total identifications for each treatment) was compared, relative to the strain, cellular growth phase, and pH of the growth media (Figure 4.2). Increased protein abundances in both the persistent control and persistent factory *L. monocytogenes* were identified relative to the sporadic factory contaminant using statistical analyses. Strain level variation on a protein functional level was most noticeable in exponential and stationary growth phase at pH 8.5 (Figure 4.2).

The number of proteins associated with biosynthesis of amino acids increased for all strains in stationary growth phase at pH8.5, particularly the persistent control strain 102-195-242, while remaining constant between growth phases at pH7.3. Similarly, proteins involved in fatty acid / phospholipid metabolism increased in abundance between exponential and stationary growth phase under both pH7.3 and pH8.5 culture conditions, with the exception of the sporadic strain DS_14, which presented a decreased abundance between these growth phases at pH7.3.

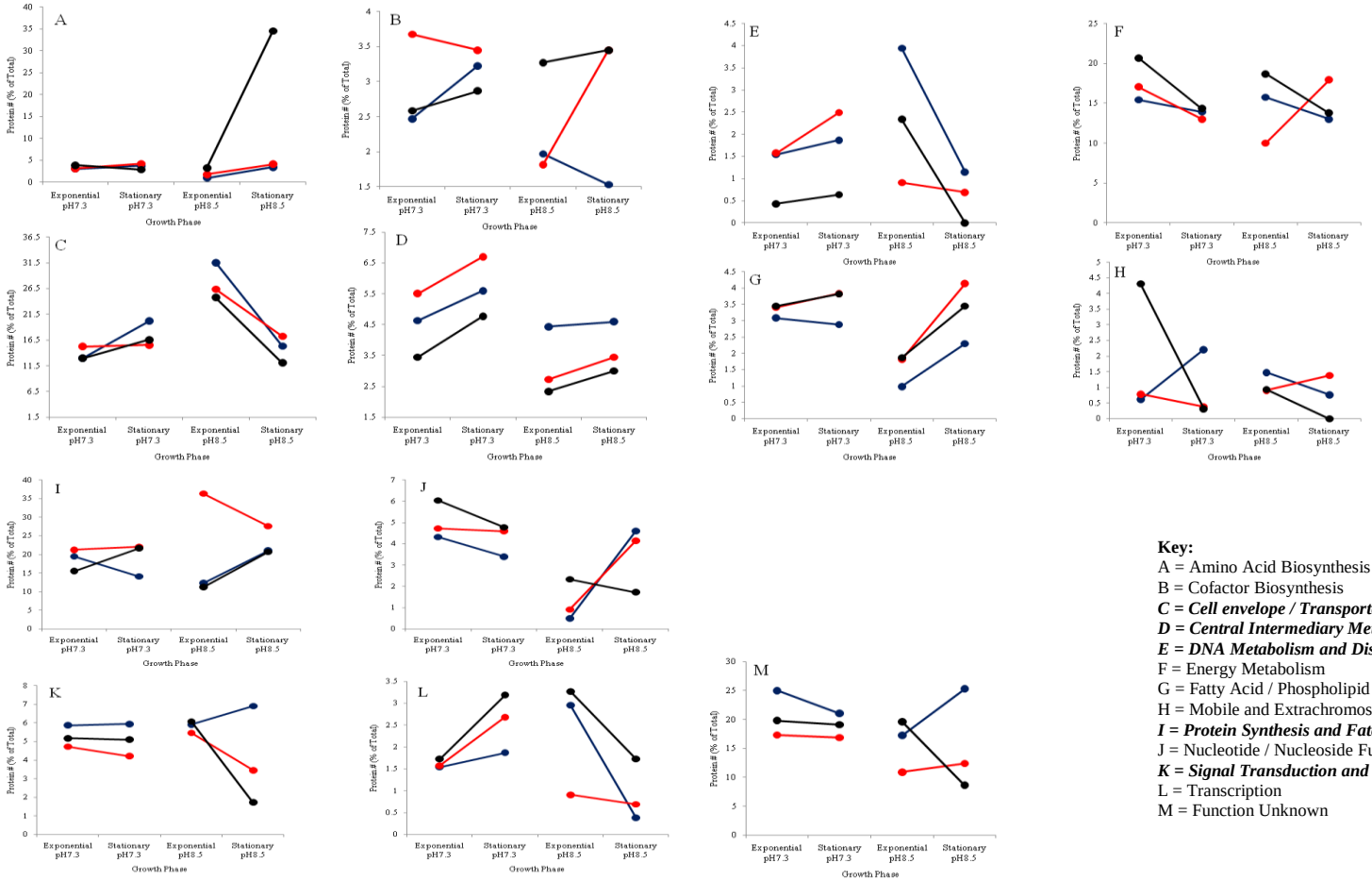
Proteins associated with central intermediary metabolism and cellular processes increased in abundance for all strains between exponential and stationary growth, under both pH7.3, and pH8.5, culture conditions. This was most pronounced at pH7.3, and for the sporadic strain DS_14, at pH8.5. Proteins with roles in metabolism of DNA (degradation of DNA), cell envelope, transcription (degradation of RNA), transport and protein binding increased in abundance between exponential and stationary growth phases at pH7.3, while decreased abundance of these elements was observed between these two states at pH8.5.

Abundance of proteins with signal transduction and regulatory functions remained constant for all strains, independent of growth phase, at pH7.3. However, at pH8.5, a decreased abundance of these proteins was observed for the persistent *L. monocytogenes* strains between exponential and stationary growth phase, while an increase was observed for the sporadic strain DS_14. A similar pattern was observed for proteins involved in biosynthesis of cofactors at pH8.5, with increased abundance noted for the persistent strains between exponential and stationary growth phase, coupled with a decrease in abundance for the sporadic strain DS_14.

Proteins associated with mobile and extra-chromosomal elements (which include phage and viral functions) decreased from exponential to stationary growth phase, independent of the pH of the culture medium, for the persistent control strain 102-195-242. However, abundance of these proteins increased and decreased between growth states for the sporadic strain DS_14 at pH7.3 and pH8.5 respectively, with the opposite of this observed for persistent strain DS_81. The number of proteins associated with nucleoside/nucleotide functions (including salvage, repair and synthesis) decreased from exponential to stationary growth phase at pH7.3 for all strains. However, abundance of these proteins increased for the persistent and sporadic factory strain between these growth phases at pH8.5, while it decreased for the persistent control strain 102-195-242. The same pattern was observed for the abundance of proteins with unknown function.

Protein abundance associated with energy metabolism decreased between exponential and stationary growth phase for both pH7.3 and pH8.5 culture conditions, with the exception of persistent factory strain DS_81, which had an increased abundance of these proteins between growth phases at pH8.5. Finally, proteins associated with protein synthesis and fate increased in abundance for the persistent control strain between exponential and stationary growth at both pH7.3 and pH8.5,

while they were observed to decrease for the sporadic strain DS_14 at pH7.3, and increase for the persistent strain DS_81 at pH8.5.



Key:
A = Amino Acid Biosynthesis
B = Cofactor Biosynthesis
C = Cell envelope / Transporters and Binding Proteins
D = Central Intermediary Metabolism and Cellular Processes
E = DNA Metabolism and Disrupted reading Frame
F = Energy Metabolism
G = Fatty Acid / Phospholipid Metabolism
H = Mobile and Extrachromosal Element Functions
I = Protein Synthesis and Fate
J = Nucleotide / Nucleoside Functions
K = Signal Transduction and Regulatory Functions
L = Transcription
M = Function Unknown

Figure 4.2 The proportional distribution (% relative to the total protein identifications for that treatment) of protein identifications assigned to functional roles as defined by the JCVI-CMR, Genolist and KEGG functional ontology systems for *L. monocytogenes.* Strains (DS_14 (blue), DS_81 (red) and 102-195-242 (black)) were compared according to growth phase and pH of the growth media. Note: In some instances, the functional assignment codes outlined in Table 4.2 were pooled based on similarity of biological role for ease of presentation. This is detailed in the key by bold italic font.

4.3.2 Comparison of protein abundances recovered from environmentally persistent relative to sporadic *L. monocytogenes* strains

Spectral abundance (relative protein quantitation) of the persistent *L. monocytogenes* strains 102-195-242 (control), and DS_81 (persistent factory contaminant) was compared in exponential and stationary growth phase at pH7.3 and pH8.5 to sporadic strain DS_14. Only proteins found in all three strains were quantitatively compared on the basis of normalised spectral counts.

4.3.2.1 pH7.3 versus pH7.3

4.3.2.1.1 Exponential growth phase

The persistent *L. monocytogenes* strains, 102-195-242 (control), and DS_81 (persistent factory contaminant), presented significantly increased abundance of 82 proteins ($G_{adj}>3.841$; χ^2, 1df; $p<0.05$) relative to the sporadic factory contaminant DS_14 in exponential growth phase at pH7.3 (Table 4.3). Amongst these were three proteins (AroK, AroD, AroH) associated with the biosynthesis of aromatic amino acids, a "YceI-like" family protein (associated with menaquinone/ubiquinone biosynthesis and stress response in *L. monocytogenes*; Abram *et al.*, 2008b), a protein associated with the biosynthesis and degradation of polysaccharides, and five proteins associated with chemotaxis and motility, detoxification, pathogenesis, and adaptation to atypical conditions. Abundance of fifteen proteins associated with central intermediary (n = 5), and energy (n = 10), metabolism increased. The proteins associated with central intermediary metabolism had amino-sugar, sulphur and "other" (two unknown and a phosphotransacetylase) functions, while those involved with energy metabolism had roles in ATP production-proton motive force, the electron transport chain, fermentation, and glycolysis – gluconeogenesis. Increased abundance of four proteins functioning in the biosynthesis of fatty acids was identified, coupled to a single protein functioning in fatty acid degradation, while abundance of proteins involved in protein folding, stability, modification and repair were increased, along with a single protein involved in transport and binding. Three regulatory proteins showed increased abundance, including two associated with DNA response regulation, and a GTP-binding protein.

Multiple proteins associated with protein synthesis were present in increased abundance, consisting predominantly of proteins involved in ribosome synthesis and modification (n = 14), as well as tRNA amino acids (n = 1), translation factors (n = 2) and "other" (a peptidyl-tRNA hydrolase) functions. Adenylate kinase, functioning in nucleoside interconversion, and a purine nucleoside phosphorylase, functioning in the salvage of nucleotides were more abundant.

4.3.2.1.2 Stationary growth phase

In stationary growth phase, at pH7.3, the persistent *L. monocytogenes* strains presented 97 proteins with significantly increased abundances (G_{adj}>3.841; χ^2, 1df; $p<0.05$) relative to the sporadic strain DS_14 (Table 4.3). Increased abundance of proteins associated with aromatic amino acid, ubiquinone (napthoate synthase), aspartate and serine biosynthesis was detected, as was abundance of cell envelope proteins functioning in metabolism of peptidoglycan, polysaccharides, chemotaxis/motility, detoxification, and proteins associated with stress adaptation.

Proteins with increased abundance in the persistent strains that are associated with central intermediary metabolism were similar (in both number and functional categorisation) to those observed in exponential growth phase at pH7.3. Similarly, in stationary phase, the number of proteins identified with increased abundance that are associated with energy production remained similar to observations in exponential phase at pH7.3, however their specific functions differed. Abundance of proteins associated with ATP-proton motive force, the electron transport chain, fermentation and glycolysis – gluconeogenesis increased in the persistent strains, as did proteins associated with aerobic, amino acid and amine, the pentose phosphate pathway, sugars, the TCA cycle and methanogenesis related energy production.

A single protein associated with fatty acid metabolism was more abundant, likely part of an energy generating pathway based on the functional roles allocated, while multiple proteins associated with protein synthesis had increased abundance in the persistent strains. These almost exclusively served in ribosome synthesis and modification, along with tRNA amino acids, translation initiation and elongation factors. Proteins associated with protein fate and regulation were more abundant, including those involved in peptide secretion and trafficking, modification and repair of proteins, protein/peptide/glycoprotein degradation, and plasmid-transformation processes, as well as transcriptional repression and DNA compaction/replication.

Additionally, transport and binding proteins associated with transcription, carbohydrates/alcohols and acids were more abundant, as were proteins associated with purines, pyrimidines, nucleosides and nucleotides, which fulfilled similar roles (nucleoside interconversion and nucleotide salvage) to those detected in exponential growth phase at pH7.3.

Table 4.3 Significantly different protein abundances (G_{adj}; χ^2 >3.841, p≤0.05) identified by comparison of both of the environmentally persistent *L. monocytogenes* strains (102-195-242* and DS_81) with the environmentally sporadic *L. monocytogenes* strain DS_14 during growth at pH7.3.

Growth Phase (G_{adj})				Functional Category		Protein	ORF Name	Protein Description
Exponential		Stationary						
102*	DS_81	102*	DS_81	Major	Minor			
17.5	29.8	28.3	24.5	A	1	AroK	lmo1749	Shikimate kinase
7.2	8.1	-	-	A	1	AroD	lmo0491	3-dehydroquinate dehydratase, type I
8.3	8.8	6.4	6.2	A	1	AroH	lmo1926	Chorismate mutase
-	-	9.9	14.1	A	2	ThrC	lmo2546	Threonine synthase
-	-	4.5	9.9	A	5	GlyA	lmo2539	Hydroxymethyltransferase
-	-	5.2	24.9	B	10	MenB	lmo1673	Naphthoate synthase
5.3	8.0	-	-	B	10	-	lmo0796	YceI like family protein
-	-	8.1	9.3	C	20	MreB	lmo1713	Cell shape determining protein
-	-	5.2	5.1	C	20	PgdA	lmo0415	Peptidoglycan deacetylase
29.4	25.5	19.6	14.8	C	21	RfbD	lmo1084	dTDP-4-dehydrorhamnose reductase
-	-	4.1	4.0	C	22	-	lmo1799	Putative peptidoglycan bound protein (LPXTG motif)
-	-	4.1	4.0	D	24	FliF	lmo0713	Flagellar MS-ring protein
5.0	5.3	-	-	D	24	FlgC	lmo0711	Flagellar basal-body rod protein
26.5	31.8	7.4	7.3	D	25	Sod	lmo1349	Superoxide dismutase
4.0	4.2	-	-	D	28	-	lmo0394	P60 extracellular protein
-	-	18.8	52.4	D	29	-	lmo1580	Universal stress protein family
4.2	12.0	-	-	D	29	-	lmo0515	Universal stress protein family
7.2	7.6	-	-	D	29	CspLB	lmo2016	Cold shock-like protein
-	-	51.0	60.8	D	27,25	ArsC	lmo2426	Glutaredoxin
18.8	19.9	23.3	19.7	R	91	-	lmo0783	PTS system, mannose/fructose/sorbose family, IIB component subfamily
-	-	4.7	19.2	E	36	Ndh	lmo2389	Oxidative phosphorylation, NADH dehydrogenase

-	-	6.4	5.2	E	36	-	lmo2444	Carbohydrate metabolism
-	-	4.1	4.0	E	36	-	lmo2446	Carbohydrate metabolism
16.3	16.7	-	-	E	31	-	lmo2358	Sugar metabolism
28.2	30.0	27.1	26.2	E	34	-	lmo0609	Rhodanese-like domain protein
6.1	10.1	-	-	E	36	-	lmo1736	Acetyltransferase
18.8	19.9	6.5	10.0	E	36	-	lmo0938	Protein-tyrosine phosphatase
-	-	5.2	22.0	H	42	Ldh	lmo0210	L-lactate dehydrogenase
-	-	5.2	22.0	H	45	AtpB	lmo2535	ATP synthase F1, beta subunit
4.9	6.0	-	-	H	45	AtpC	lmo2528	ATP synthase F1, epsilon subunit
16.5	17.8	4.1	4.4	H	46	-	lmo1609	Thiredoxin family protein; folding catalyst
21.1	49.1	12.3	45.9	H	48	PflA	lmo1917	Formate acetyltransferase
7.4	467.6	-	-	H	49	FbaA	lmo2556	Fructose-bisphosphate aldolase
-	-	12.0	53.8	H	49	PykA	lmo1570	Pyruvate kinase
12.6	8.0	54.1	63.3	H	49	Pgk	lmo2458	Phosphoglycerate kinase
-	-	4.1	4.0	H	49	Fruk	lmo2095	Carbohydrate metabolism
13.2	62.5	11.5	11.7	H	49	Eno	lmo2455	Enolase
6.9	8.4	-	-	H	49	-	lmo0556	Similar to phosphoglycerate mutase
-	-	12.0	10.8	H	50	Tkt	lmo1305	Transketolase
4.7	32.8	52.4	79.5	H	50	Gnd	lmo1376	6-phosphogluconate dehydrogenase
-	-	4.1	4.0	H	52	GmaR	lmo0688	Carbohydrate metabolism
-	-	5.8	21.6	H	52	LacD	lmhcc_2092	Tagatose 1,6-diphosphate aldolase
4.0	19.7	5.9	10.8	H	52	PfkB	lmo2095	Fructose-1-phosphate kinase
-	-	4.1	4.0	H	52	RhaA	lm4b_02820	L-rhamnose isomerase
14.1	13.4	-	-	H	52	GclH	lmo2425	Glycine cleavage system H protein
-	-	4.1	5.5	H	53	PptA	lmo2564	4-oxalocrotonate tautomerase family protein
-	-	4.1	10.4	H	54	FolD	lmo1360	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase

-	-	5.1	4.2	I	58,52	DkaL	lmo2696	Dihydroxyacetone kinase, Dak2 subunit
45.9	240.4	-	-	E	48,36	Pta	lm4b_02124	Phosphotransacetylase
4.4	18.8	-	-	I	58,52	DhaK	lmo0348	Dihydroxyacetone kinase
7.0	25.0	-	-	I	58	FabG	lmo1807	3-oxoacyl-(acyl-carrier-protein) reductase
15.9	24.0	-	-	I	58	FabD	lmhcc_0749	Acyl-carrier-protein S-malonyltransferase
4.0	6.9	-	-	I	58	PlsX	lm4b_01825	Fatty acid/phospholipid synthesis protein
4.0	12.6	-	-	I	59	-	lmo2452	Carboxylesterase
15.7	4.6	8.2	4.3	L	61,26	OppA	lmo2196	Oligopeptide ABC transporter, oligopeptide-binding protein
3.8	10.1	7.9	9.2	L	64	FtsY	lmo1803	Cell division ABC transporter, substrate-binding protein
-	-	6.4	6.2	L	98,67	P45	lmo2321	Peptidoglycan lytic protein P45
-	-	16.5	16.5	L	64	-	lmo1739	ATP dependant transporter
12.8	75.6	56.2	55.2	L	65	-	lmo0286	Methionine aminopeptidase
10.0	75.1	-	-	L	66	Tig	lmo1267	Trigger factor
8.4	17.4	-	-	L	66	GroES	lmo2069	Co-chaperonin GroES
6.8	13.4	-	-	L	66	DnaK	lmo1473	Class I heat-shock protein (molecular chaperone)
21.1	21.2	-	-	M	68	GltX	lmo0237	Glutamyl-tRNA synthetase
-	-	6.5	26.0	M	68	GatB	lmhcc_0810	Glutamyl-tRNA (Gln) amidotransferase, B subunit
-	-	5.2	31.3	M	68	SerS	lmhcc_2785	Seryl-tRNA synthetase
-	-	4.1	4.0	M	68	GlyQ	lmof2365_1478	Glycyl-tRNA synthetase subunit alpha
28.0	43.7	-	-	M	69	RpsH	lmo2618	30S ribosomal protein S8
-	-	4.9	9.0	M	69	RpsJ	lmo2633	30S ribosomal protein S10
9.2	36.9	15.9	8.9	M	69	RpsB	lmo1658	30S ribosomal protein S2
20.2	23.6	-	-	M	69	RplL	lmo0251	50S ribosomal protein L7/L12
-	-	60.4	11.8	M	69	RpmA	lmo1540	50S ribosomal protein L27
-	-	18.0	25.9	M	69	RplW	lmo2630	50S ribosomal protein L23
-	-	6.8	6.6	M	69	RplX	lmo2621	50S ribosomal protein L24

-	-	10.6	10.8	M	69	RplQ	lmo2605	50S ribosomal protein L17
6.2	9.2	-	-	M	69	RplJ	lmo0250	50S ribosomal protein L10
4.0	4.6	-	-	M	69	GidB	lmo2802	Glucose-inhibited division protein B
8.3	7.8	-	-	M	69	RpsC	lmo2626	30S ribosomal protein S3
27.0	23.9	24.6	20.1	M	69	RpmF	lmo0486	50S ribosomal protein L32-2
31.8	33.8	-	-	M	69	RpsQ	lmo2623	30S ribosomal protein S17
-	-	6.0	6.4	M	69	RplM	lmo2597	50S ribosomal protein L13
-	-	19.6	19.4	M	69	RpsP	lmo1797	30S ribosomal protein S16
5.0	5.3	-	-	M	69	RpsL	lmo2656	30S ribosomal protein S12
29.4	31.2	-	-	M	69	RpmJ	lmo2609	50S ribosomal protein L36
-	-	4.1	4.7	M	69	RplU	lmo1542	50S ribosomal protein L21
-	-	15.5	24.1	M	69	RplA	lmo0249	50S ribosomal protein L1
-	-	27.8	63.6	M	69	RpmD	lmo2614	50S ribosomal protein L30
7.2	7.6	8.7	6.8	M	69	RpmB	lmo1816	50S ribosomal protein L28
-	-	5.2	6.3	M	69	RplR	lmo2616	50S ribosomal protein L18
23.5	24.9	63.7	43.6	M	69	RpsT	lmo1480	30S ribosomal protein S20
21.1	22.4	-	-	M	69	RpsU	lmo1469	30S ribosomal protein S21
4.0	4.2	-	-	M	69	-	lmo0216	S4 domain protein, similar to heat shock protein
-	-	8.0	9.0	M	71	InfA	lmo2610	Translation initiation factor IF-1
-	-	10.0	9.3	M	71	InfC	lmo1785	Translation initiation factor IF-3
4.9	181.6	79.0	161.0	M	71	Fus	lmo2654	Elongation factor G
-	-	157.4	81.1	M	71	TufA	lmo2653	Elongation factor Tu
6.3	70.9	-	-	M	71	Tsf	lmo1657	Translation elongation factor Ts
8.3	7.4	-	-	M	72	Pth	lmo0213	Peptidyl-tRNA hydrolase
35.8	39.5	5.5	4.9	N	74	Adk	lmo2611	Adenylate kinase
-	-	5.2	4.3	N	75	PlcA	lmo0201	Inositol-5-monophosphate dehydrogenase

8.3	7.2	5.8	5.2	N	77	Pnp	Lmo1331	Purine nucleoside phosphorylase
-	-	10.4	9.6	N	77	-	-	Fusion protein, similarity to *B. subtilis* YacA
-	-	11.4	6.9	O	79	Hup	lmo1934	DNA-binding protein HU
4.2	6.2	8.4	8.1	O	82	-	lmo1022	DNA-binding response regulator
9.5	7.9	-	-	O	82	EngB	lm4b_01569	GTPase, GTP-binding protein
-	-	6.0	23.0	O	82	CodY	lmo1280	Transcriptional repressor CodY
10.6	11.2	5.2	5.1	O	82	-	lmo0749	Transcription regulator
-	-	5.2	4.4	R	88	MetS	lmo0177	Methionyl-tRNA synthetase
8.3	8.8	-	-	S	96	-	lmo0212	Similar to acetyltransferase
17.6	18.6	-	-	S	96	-	lmo1240	Similar to phosphoesterase
-	-	14.8	53.3	S	97	TypA	lmo1067	GTP-binding protein
-	-	20.2	24.7	S	97	-	lmo2216	HIT family protein
9.5	10.0	4.1	4.0	S	97	EbsC	lmo0790	Hypothetical protein
8.9	9.9	-	-	S	99	-	lmo0579	Hypothetical protein
31.6	19.8	4.2	35.0	S	99	-	lmo1249	Hypothetical protein, contains a haemagglutination activity site
7.5	7.7	-	-	S	99	-	lmo0369	Hypothetical protein, similar to *B. subtilis* YeeI protein (phosphotransferase)
-	-	6.4	5.1	S	99	-	lmo1453	Hypothetical protein. Transposon insertion into orfA impairs growth/virulence
25.9	20.8	-	-	S	99	-	lmo0592	Hypothetical protein, has a motif similar to membrane bound haemolysin
51.1	38.3	-	-	S	99	-	lmo2703	Hypothetical protein
-	-	4.1	4.0	S	99	-	lmo2729	Hypothetical protein
3.8	4.1	-	-	S	99	-	lmo1242	Hypothetical protein, similar to *B. subtilis* YdeI protein (peptide antimicrobial resistance)
9.5	8.0	6.8	6.2	S	99	-	lmo1283	Hypothetical protein, carbohydrate metabolism
6.5	9.0	17.2	16.6	S	99	-	lmo2759	Hypothetical protein, glucose metabolism
-	-	5.2	5.1	S	99	-	lmo2579	Hypothetical protein, antibiotic biosynthesis
5.0	11.2	-	-	S	99	-	lmo2048	Hypothetical protein
9.5	8.3	9.9	9.6	S	99	-	lmo0718	Hypothetical protein

48.6	39.7	35.8	34.7	S	99	-	lmo0903	Hypothetical protein, oxidative stress
7.2	7.6	6.4	6.2	S	99	-	lmo0102	Hypothetical protein, antibiotic biosynthesis
6.1	6.5	-	-	S	99	-	-	Putative uncharacterised protein, strain J0161
19.9	21.1	29.6	28.6	S	99	-	lmo1059	Hypothetical protein, contains a thioredoxin domain, folding catalyst
-	-	24.2	27.3	S	99	-	lmo2223	Hypothetical protein
-	-	6.4	4.8	S	99	-	lmo0289	Hypothetical protein
-	-	6.4	4.3	S	99	-	lmo0794	Hypothetical protein
-	-	21.9	18.7	S	99	-	lmo1214	Hypothetical protein
-	-	6.4	4.8	S	99	-	lmo1510	Hypothetical protein
-	-	5.2	5.1	S	99	-	lmo0957	Hypothetical protein
-	-	37.3	25.5	S	99	-	lmo2158	Hypothetical protein
17.6	18.6	12.3	9.3	S	99	-	lmo1323	Hypothetical protein, transcription regulation
48.6	51.7	5.2	5.1	S	99	-	lmo1659	Hypothetical protein
5.0	5.3	-	-	S	99	-	lmo1863	Hypothetical protein, contains a DegV domain, likely associated w/ fatty acid transport
-	-	13.5	11.7	S	99	-	lmo1503	Hypothetical protein
4.0	5.7	-	-	S	99	-	lmo0056	Hypothetical protein, heat shock protein
-	-			S	99	-	lmo2457	Hypothetical protein
4.0	5.7	-	-	S	99	-	-	Putative uncharacterised protein, strain J0161

4.3.2.2 pH8.5 versus pH7.3

4.3.2.2.1 Exponential growth phase

In exponential growth phase at pH8.5, the persistent *L. monocytogenes* strains, 102-195-242 (control), and DS_81 (persistent factory contaminant), presented only two proteins with significantly different abundance (G_{adj}>3.841; χ^2, 1df; p<0.05) relative to the sporadic factory contaminant DS_14 grown at pH7.3. These were cold shock-like protein (CspLB), involved in adaptation to atypical conditions (DNA dependent (binding) regulation of transcription), and elongation factor Tu (EF-Tu), associated with translation; specifically, the binding and transport of the appropriate codon-specified aminoacyl-tRNA to the aminoacyl site of the ribosome.

4.3.2.2.2 Stationary growth phase

In stationary growth phase at pH8.5 the persistent *L. monocytogenes* strains, 102-195-242 (control), and DS_81 (persistent factory contaminant), 24 proteins were significantly more abundant (G_{adj}>3.841; χ^2, 1df; p<0.05) than in the sporadic factory contaminant DS_14 grown at pH7.3 (Table 4.4). Again, proteins associated with aromatic amino acid biosynthesis and an "YceI-like" family protein were increased. Abundance of proteins associated with ribosome synthesis and modification was also increased.

Cell envelope proteins that were more abundant included glucose-1-phosphate thymidylyltransferase and dTDP-4-dehydrorhamnose reductase (both involved in polyketide sugar unit, teichoic acid and streptomycin biosynthesis), as well as a putative peptidoglycan-bound protein (containing a LPXTG motif) and a transmembrane protein of unknown function. Proteins associated with the fate of other proteins with increased abundance included Co-chaperonin GroES, peptidoglycan lytic protein P45, and a cell-division associated transporter.

Single proteins associated with energy metabolism and transport/binding were more abundant including the flagellar biosynthesis antirepressor (lmo0688) and PTS subunit IIA, putatively specific to glucose (lmo1017).

Table 4.4 Significantly different protein abundances (G_{adj}; χ^2 >3.841, p≤0.05) between the environmentally persistent, *L. monocytogenes* strains (102-195-242* and DS_81) and an environmentally sporadic *L. monocytogenes* strain DS_14 during stationary growth at pH8.5 relative to pH7.3.

Strain (G_{adj})		Functional Category		Protein	ORF Name	Protein Description
102*	DS_81	Major	Minor			
26.5	11.9	A	1	AroK	lmo1749	Shikimate kinase
10.8	5.2	B	10	-	lmo0796	YceI like family protein
6.2	4.3	C	21	RfbA	lmo1081	Glucose-1-phosphate thymidylyltransferase
13.2	6.6	C	21	RfbD	lmo1084	dTDP-4-dehydrorhamnose reductase
14.4	6.8	C	22	-	lmo1799	Putative peptidoglycan bound protein (LPXTG motif)
12.0	5.8	S	99	-	-	Putative transmembrane protein, strain J0161
11.3	10.1	R	91	Crr	lmo1017	PTS system, glucose specific IIA component
8.5	4.2	D	24	GmaR	lmo0688	Flagellar biosynthesis antirepressor
13.2	5.7	L	98,67	P45	lmo2321	Peptidoglycan lytic protein P45
10.4	5.0	L	64	FtsY	lmo1803	Cell division ABC transporter, substrate-binding protein
12.4	15.2	L	66	GroES	lmo2069	Co-chaperonin GroES
8.5	4.2	M	69	RpsL	lmo2656	30S ribosomal protein S12
62.9	31.1	M	69	RpsH	lmo2618	30S ribosomal protein S8
6.2	17.1	M	69	RplK	lmo0248	50S ribosomal protein L11
20.6	9.4	M	69	RplW	lmo2630	50S ribosomal protein L23
17.0	7.3	M	69	RplF	lmo2617	50S ribosomal protein L6
14.9	11.2	M	69	RpsQ	lmo2623	50S ribosomal protein L17
7.2	5.7	M	69	RplM	lmo2597	50S ribosomal protein L13
16.8	7.4	M	69	RpsP	lmo1797	30S ribosomal protein S16
20.4	9.3	S	99	-	lmo0903	OsmC family protein
19.2	8.8	S	99	-	lmo1059	Contains a thioredoxin domain, folding catalyst
17.2	8.6	S	99	-	lmo1283	Hypothetical protein, carbohydrate metabolism
12.0	5.8	S	99	-	lmo1323	Hypothetical protein, transcription regulation
15.6	7.3	S	99	-	lmo1659	Hypothetical protein

4.3.2.3 pH8.5 versus pH8.5

4.3.2.3.1 Exponential growth phase

In exponential growth phase at pH8.5, 13 proteins were significantly more abundant in the persistent *L. monocytogenes* strains, 102-195-242 (control), and DS_81 (persistent factory contaminant) relative to the sporadic factory contaminant DS_14 (G_{adj}>3.841; χ^2, 1df; p<0.05; Table 4.5). These included chemotaxis protein CheY, cold shock proteins CspLA and CspLB (associated with adaptation to atypical conditions), ribosomal synthesis and modification proteins and hypothetical protein lmo0171 (a peptidoglycan bound cell surface protein of unknown function). Finally, increased abundance of the energy metabolism proteins thioredoxin (electron

transport chain), fructose bisphosphate aldolase and phosphoglycerate kinase (glycolysis – gluconeogenesis) were observed.

4.3.2.3.2 Stationary growth phase

In stationary growth phase at pH8.5, 75 proteins were significantly differently expressed in the persistent *L. monocytogenes* strains, 102-195-242 (control), and DS_81 (persistent factory contaminant), (G_{adj}>3.841; χ^2, 1df; p<0.05) relative to the sporadic factory contaminant DS_14 (Table 4.5). These included proteins associated with aromatic amino acid biosynthesis and a "YceI-like" family protein that were more abundant, as were the cell envelope proteins glucose-1-phosphate thymidylyltransferase and dTDP-4-dehydrorhamnose reductase, with the addition of D-alanine—poly(phosphoribitol) ligase subunit 2 (involved in teichoic acid biosynthesis and able to affect cell wall charge). Proteins associated with cellular processes that were more abundant included CspLA, a surface antigen (CD4+ T-cell stimulator) and a superoxide dismutase (protects against oxidative stress). Transport and binding proteins were increased overall, with significantly increased abundance (relative to DS_14) of ATP binding ABC transporters, phosphocarrier protein Hpr, PTS system (cellubiose specific, IIB component), OppA (an oligopeptide ABC transporter), and hypothetical protein lmo1017 (involved in glycolysis – gluconeogenesis).

Increased abundance of proteins associated with central intermediary metabolism was detected, including a protein of the Cof – like hydrolase superfamily and four hypothetical proteins associated with amino – sugar and nucleotide sugar metabolism (lmo2358), oxidation/reduction (lmo0823), starch and sucrose metabolism (lmo2831), and a protein – tyrosine phosphatase (lmo0938).

Proteins associated with ribosomal synthesis and modification were again present in both increased number (n = 17), and abundance, along with translation initiation (IF-3) and elongation factors (EF-Tu, EF-Ts, GreA), and a ribosome recycling factor. Additionally, the transcription associated proteins NusG (associated with transcription antitermination) and RsbT (an anti – sigma B factor) were more abundant. Increased numbers of proteins associated with the fate of other proteins was observed, with increased abundance of proteins associated with peptide secretion and trafficking, degradation of proteins / peptides / glycoproteins, folding and stability, and protein modification and repair. The regulatory proteins DNA-binding

HU, ResD (a DNA-binding response regulator), a peptidoglycan bound protein with an LPXTG motif, along with adenylate kinase (involved in nucleotide interconversion) and uracil phosphor-ribosyltransferase (involved in nucleotide salvage) were also more abundant. A single protein involved in fatty acid biosynthesis was more abundant, while five proteins associated with general energy metabolism were detected in increased amounts, including lmo1609 (electron transport chain), pyruvate kinase, phosphopentomutase, and a glycosyltransferase.

Table 4.5 Significantly different protein abundances (G_{adj}; χ^2 >3.841, p≤0.05) identified by comparison of both of the environmentally persistent *L. monocytogenes* strains (102-195-242* and DS_81) with the environmentally sporadic *L. monocytogenes* strain (DS_14) during growth at pH8.5.

Growth Phase (G_{adj})				Functional Category		Protein	ORF Name	Protein Description
Exponential		Stationary						
102*	DS_81	102	DS_81	Major	Minor			
-	-	18.370	13.447	A	1	AroK	lmo1749	Shikimate kinase
-	-	6.630	5.324	A	1	AroD	lmo0491	3-dehydroquinate dehydratase, type I
-	-	7.445	5.901	B	10	-	lmo0796	YceI like family protein
-	-	9.092	7.059	C	20	DdlA	lmo0855	D-alanine--poly(phosphoribitol) ligase subunit 2
4.6	4.9	-	-	C	22	-	lmo0171	Gram positive anchored protein
-	-	4.235	4.850	C	21	RfbA	lmo1081	Glucose-1-phosphate thymidylyltransferase
-	-	9.092	7.406	C	21	RfbD	lmo1084	dTDP-4-dehydrorhamnose reductase
5.0	4.6	-	-	D	24	CheY	lmo0691	Chemotaxis protein
-	-	5.023	4.172	D	25	Sod	lmo1349	Superoxide dismutase
-	-	5.023	4.172	D	28	TscA	lmo1388	CD4+ T cell-stimulating antigen, lipoprotein
13.2	12.1	13.520	11.382	D	29	CspLA	lmo1879	Cold shock-like protein A
14.3	13.5	-	-	D	29	CspLB	lmo2016	Cold shock-like protein B
-	-	17.517	12.865	R	91	Crr	lmo1017	Hypothetical protein, PTS system, glucose specific IIA component
-	-	21.793	15.396	R	91	PtsH	lmo1002	Phosphocarrier protein
-	-	4.235	3.904	R	95	SufC	lmo2415	Fe-S assembly protein, ATP-binding
-	-	4.235	9.746	R	95	MetN	lmo2419	D-methionine ABC transporter, ATP-binding protein
-	-	30.737	25.779	R	91,51,1	-	lmo2373	PTS system, cellobiose-specific, IIB component
-	-	26.091	17.186	E	31	-	lmo2358	Hypothetical protein, amino-sugars metabolism
-	-	9.922	7.068	E	36	-	lmo0663	Cof-like hydrolase
-	-	5.822	4.747	E	36	-	lmo0823	Hypothetical protein, similar to aldo/keto reductase
-	-	6.630	5.324	E	36	-	lmo0938	Hypothetical protein, signal transduction

-	-	5.023	3.849	E	36	-	lmo2831	Hypothetical protein, starch/sucrose metabolims
-	-	5.023	4.172	H	46	-	lmo1609	Hypothetical protein, carbohydrate metabolism
10.8	12.5	-	-	H	46	TrxA	lm01233	Thioredoxin
4.9	10.9	-	-	H	49	FbaA	lmo2556	Fructose bisphosphate aldolase
4.2	6.0	-	-	H	49	Pgk	lmo2458	Phosphoglycerate kinase
-	-	4.235	14.938	H	49	PykA	lmo1570	Pyruvate kinase
-	-	8.266	5.956	H	52	Drm	lmo1954	Phosphopentomutase
-	-	5.822	4.747	D	24	GmaR	lmo0688	Hypothetical protein
-	-	8.266	6.639	I	58	FabD	lmhcc_0749	Acyl-carrier-protein S-malonyltransferase
-	-	4.235	5.425	K	61,26	OppA	lmo2196	Oligopeptide ABC transporter, oligopeptide-binding protein
-	-	9.092	6.468	L	98,67	P45	lmo2321	Peptidoglycan lytic protein P45
-	-	8.266	6.071	L	64	FtsY	lmo1803	Cell division ABC transporter, substrate-binding protein
-	-	6.630	9.247	L	65	-	lmo0286	Methionine aminopeptidase, type I
-	-	9.092	22.858	L	66	Tig	lmo1267	Trigger factor
-	-	26.953	19.266	L	66	GroES	lmo2069	Co-chaperonin GroES
-	-	5.023	4.303	L	66	DnaK	lmo1473	Class I heat-shock protein
-	-	5.822	4.479	M	69	-	-	Putative uncharacterized protein, strain J0161
-	-	5.822	4.747	M	69	RpsL	lmo2656	30S ribosomal protein S12
-	-	15.816	11.703	M	69	RpsJ	lmo2633	30S ribosomal protein S10
-	-	7.445	5.901	M	69	RpsS	lmo2628	30S ribosomal protein S19
-	-	80.860	57.533	M	69	RpsH	lmo2618	30S ribosomal protein S8
18.4	19.4	45.853	61.033	M	69	RplK	lmo0248	50S ribosomal protein L11
-	-	37.333	24.480	M	69	RpsQ	lmo2617	50S ribosomal protein L6
-	-	14.967	12.551	M	69	RpsE	lmo2615	30S ribosomal protein S5
-	-	19.224	14.029	M	69	RpsQ	lmo2623	50S ribosomal protein L17
-	-	16.666	11.474	M	69	RplM	lmo2597	50S ribosomal protein L13

4.9	5.1	11.594	8.334	M	69	RpsP	lmo1797	30S ribosomal protein S16
-	-	9.922	17.775	M	69	RpsB	lmo1658	30S ribosomal protein S2
-	-	6.630	9.940	M	69	RplL	lmo0251	50S ribosomal protein L7/L12
-	-	9.092	6.576	M	69	RpsF	lmo0044	30S ribosomal protein S6
15.5	18	-	-	M	69	RpmA	lmo1540	50S ribosomal protein L27
-	-	26.953	18.317	M	69	RplP	lmo2625	50S ribosomal protein L16
-	-	20.079	13.741	M	69	RplN	lmo2622	50S ribosomal protein L14
-	-	8.266	8.468	M	69	RplE	lmo2620	50S ribosomal protein L5
-	-	16.666	11.302	M	71	InfC	lmo1785	Translation initiation factor IF-3
-	-	22.651	15.192	M	71	GreA	lmo1496	Transcription elongation factor greA
-	-	12.956	35.078	M	71	TufA	lmo2653	Elongation factor Tu
-	-	5.258	20.311	M	71	Tsf	lmo1657	Elongation factor Ts
-	-	14.121	10.926	M	71	Frr	lmo1314	Hypothetical protein Ribosome recycling factor
-	-	9.922	7.638	N	74	Adk	lmo2611	Hypothetical protein Adenylate kinase
-	-	6.630	7.950	N	77	PyrR	lmo1840	Uracil phosphoribosyltransferase
-	-	61.709	41.795	O	79	Hup	lmo1934	DNA-binding protein HU
-	-	5.023	4.172	O	82	ResD	lmo1948	DNA-binding response regulator
-	-	9.922	7.638	O	82	-	lmo1799	Putative peptidoglycan bound protein (LPXTG motif)
-	-	10.756	7.568	Q	86	NusG	lmo0246	Transcription antitermination
-	-	9.922	7.638	Q	86	RsbT	lmo0891	Hypothetical protein, anti-sigma B factor
-	-	6.630	5.324	S	96	-	lmo0212	Hypothetical protein, acetyltransferase
-	-	6.630	5.324	S	97	-	lmo2216	Hypothetical protein, HIT family protein
-	-	8.266	6.480	S	99	-	lmo0579	Hypothetical protein
-	-	5.023	3.858	S	99	-	lmo0592	Hypothetical protein, has a motif similar to a membrane bound haemolysin
-	-	14.121	10.540	S	99	-	lmo0903	Hypothetical protein, oxidative stress, OsmC protein superfamily
-	-	13.276	9.959	S	99	-	lmo1059	Hypothetical protein, contains a thioredoxin domain, folding catalyst

6.0	8.8	-	-	A,H	51,1	-	lmo2683	PTS system, cellobiose-specific, IIB component
-	-	13.276	9.959	S	99	-	lmo1283	Hypothetical protein, carbohydrate metabolism
-	-	8.266	6.480	S	99	-	lmo1323	Hypothetical protein, transcription regulation
7.9	7.5	-	-	S	99	-	lmo1252	Putative membrane protein
-	-	10.756	8.218	S	99	-	lmo1659	Hypothetical protein
-	-	20.427	19.464	S	99	-	lmo2223	Hypothetical protein
-	-	9.092	7.059	H,N	52, 73	DeoC	lmo1995	Hypothetical protein, carbohydrate, deoxyribonucleotide catabolism
5.1	7.2	-	-	S	99	SufD	lmo2414	Inorganic ion metabolism, putative Fe-S assembly
-	-	38.200	26.836	S	99	AhpC	lmo1583	Alkyl hydroperoxidase
-	-	10.756	8.218	N	78	-	lmo1502	Putative holliday junction resolvase
-	-	8.266	6.480	S	99	-	-	Transmembrane protein, strain J0161

4.4 Discussion

In this study the distribution of protein functional roles, relative to the total protein identifications, identified differences corresponding to *L. monocytogenes* strain, growth phase and pH of the culture media. However, it must be acknowledged that comparisons of this nature may be influenced by the detection efficiencies obtained for each sample during MuDPIT analysis. That is, some proteins may not be detected in fractions extracted from the same sample, even though they may actually be present, biasing proportional comparisons. This is an inherent problem with the stochastic sampling process associated with MuDPIT, generally overcome by increasing the number of fractions analysed for each biological replicate, thereby increasing sample overlap, and consequently, the likelihood of detecting most of the proteins in a given sample (Fu *et al.,* 2008; Kislinger *et al.,* 2005; Old *et al.,* 2005; Wang *et al.,* 2003; Washburn *et al.,* 2003). In comparative analysis such as the current study that are based on minimal replication and fractions per replicate, it has been demonstrated that, despite variable detections between some fractions (≤16.4% in the current study), provided that the replicate protein identifications (number of proteins corresponding to a given functional role) are summed and presented as a proportion of the total proteins for each sample, the functional distribution may still provide an overview of the qualitative protein response to a given treatment (Kislinger *et al.,* 2005; Old *et al.,* 2005). It is on this basis that the proportional distribution of proteins, corresponding to given functional roles, has been included along with the *G* – values derived from the spectral count quantitative data in this analysis.

Biosynthesis of amino acids and proteins associated with fatty acid / phospholipid metabolism appear to have a role in alkaline homeostasis, and show a level of growth phase dependence. Increased abundance of proteins associated with these functions was observed upon entry into stationary growth phase under both neutral and alkaline growth conditions, with the exception of amino acid biosynthesis proteins which remained static between growth phases at pH7.3. This was observed irrespective of strain, except for proteins associated with fatty acid/phospholipid metabolism from the "sporadic strain" which declined in number on entry to stationary phase at pH7.3.

It has been reported previously that amino acids, and the polypeptides and proteins they may go on to form, constitute an essential cytoplasmic buffering system

(Booth, 1985; Padan *et al.,* 2005; Phan – Thanh and Gormon, 1997; Giotis *et. al.,* 2008). This supports observations in the current study, in which proteins involved in biosynthesis of these components were increased. Additionally, an increase in production of these elements on transition by *L. monocytogenes* to stationary growth phase under alkaline conditions was observed. It has been shown that increased production of amino acids, specifically D – amino acids, is associated with cell wall remodelling during transition to stationary phase, and that these may also be utilised in adaptation to changing environmental conditions (Lam *et al.,* 2009). This may explain the observations in the current study, because although an increase in the number of these proteins was observed between exponential and stationary growth phase at pH7.3, the increase was most pronounced at pH8.5.

Proteins associated with amino acid production that were significantly increased under alkaline conditions in the persistent strains relative to the sporadic factory strain included those associated with aromatic amino acid biosynthesis. Aromatic amino acids have variable polarity and can result in the production of acidic by-products during the biosynthetic pathway (Alberts *et al.,* 2004). The physicochemical properties of these residues, could, therefore, contribute to net acidification of the cytoplasm as well as going on to contribute to cell wall remodelling. As such, under alkaline conditions, it could be that increased amino acid production serves an important role in stress tolerance through cytoplasmic buffering *and* cell wall remodelling superseding, or at least augmenting, the modifications associated with transition through to stationary growth phase.

It is possible that the persistent strains are capable of a more pronounced amino acid – cytoplasmic buffering and cell wall remodelling response. This could afford these strains an advantage if exposed to environmental pH fluxes, and could aid in environmental persistence. This notion is further supported by the increased number of proteins associated with fatty acid and phospholipid metabolism on transition to stationary growth phase. This was observed at both pH7.3 and 8.5, with the most pronounced shift again observed at pH8.5. Fatty acid production has previously been linked to pH stress tolerance and, as with amino acids, involved increasing production of specific isoforms that have an association with cell wall structural modification (Giotis *et al.,* 2007b). A decrease in proteins associated with fatty acid and phospholipid metabolism was observed between exponential and stationary growth phase for the sporadic factory strain, with lower numbers of these proteins at both pH in comparison to the persistent strains. Whether this represents a

diminished ability of the sporadic strain to implement physiological shifts to adapt to changing environmental conditions is unclear, but fatty acids have been implicated in the alkaline adaptation response (Giotis *et al.*, 2007b).

Further supporting the notion of alkaline induced cell wall remodeling is the observation that "YceI-like" family proteins were significantly more abundant relative to the sporadic strain at pH8.5 conditions. These proteins are characterised by a lipocalin fold; an internal cavity that functions in the binding and transport of amphiphilic molecules (Sisinni *et al.*, 2010). These proteins have been associated with sequestering of fatty acids from the external environment, and as such, may be directly contributing to cell membrane modification and cytoplasmic acidification (through importation of a surrogate proton source).

Evidence for the protein dependence of alkaline homeostasis is supported by the significantly increased abundance of proteins associated with protein stability at pH8.5. This was most pronounced in the persistent strains, and in stationary growth phase, with chaperones associated with maintenance of protein tertiary structure, as well as protein modification and repair, observed. These included co – chaperone GroES, DnaK, trigger factor, methionine aminopeptidase and substrate binding protein FtsY. Co – chaperone GroES assists the chaperone GroEL in the development and maintenance of protein folding, as does trigger factor (also associated with protein export; with both functions working in a cooperative manner with DnaK) (Deuerling *et al.*, 1999; Maier *et al.*, 2005). Methionine aminopeptidase is essential for cell growth, and is responsible for the removal of the amino-terminal (initiator) methionine from nascent proteins (Chang *et al.*, 1989; Van de Velde *et al.*, 2009). The increase in protein structural stability factors may represent a protective response induced by increased cytoplasmic pH, while the increase in methionine aminopeptidase likely reflects the observed increase in protein synthesis under pH8.5 culture conditions.

Studies have shown that FtsY is associated with membrane protein assembly, by co – translational interaction with the signal recognition particle (SRP) (e.g. Angelini *et al.*, 2005). Trigger factor binds at the same time as SRP, but is excluded by FtsY. As such, during increased expression of FtsY, membrane protein assembly may take precedence over protein export. Therefore, the presence of trigger factor probably serves predominantly protein stability functions in this instance. Notably, trigger factor has been associated with in vivo survival and persistence of *L. monocytogenes* in a mouse model (Bigot *et al.*, 2006). With this in mind,

concomitant expression of trigger factor and FtsY likely contributes to both maintenance and development of cell wall integrity and function, and increased protein structural stability, both favourable under alkaline challenge and explaining their potential role in *in vivo* persistence of *L. monocytogenes*.

Significantly increased abundance of elongation factor Tu and Ts (EF – Tu and EF – Ts) were observed in the persistent *L. monocytogenes* strains relative to the sporadic strain. EF – Tu is predominantly involved in translation elongation, while EF – Ts is associated with recycling of EF – Tu species (Kraal *et al.*, 1999). EF – Ts also has demonstrated chaperone functions, associated with maintenance of protein tertiary structure and refolding of denatured proteins (Caldas *et al.*, 1998). Based on this, it is possible that increased abundance of both EF – Tu and EF – Ts may represent a stress response, with EF – Tu assisting with protein stability, and EF – Tu maintaining the EF – Tu pool through recycling.

Protein stabilisation and cell wall modification appear to play a central role in alkaline homeostasis, and persistent strains may be better able to implement this shift under these conditions. In addition, it seems that the cell wall modification component of the alkaline adaptive response shares elements of the growth phase transition response. However, implementation of cell wall modifications cannot happen independently of other cellular processes. As such, it may be expected that a collective adaptive metabolic shift would be observed under alkaline conditions. Evidence for this response was observed in the present study.

All of the strains analysed in this study increased the number of proteins associated with both central intermediary metabolism and general cellular processes in response to the transition from exponential to stationary growth phase. Furthermore, the sporadic factory strain DS_14 presented the greatest number of these proteins, in both growth phases at pH8.5, relative to the persistent strains. The "cellular processes" functional assignment includes proteins associated with chemotaxis and motility, cell division, detoxification, pathogenesis and adaptation to atypical conditions (Table 5.2). While the number of proteins assigned to this functional role was greatest for the sporadic factory contaminant DS_14, the persistent factory contaminants (102-195-242 and DS_81) had significantly increased spectral abundances of specific elements corresponding to this group at pH8.5. This included the cold – shock proteins (CspLA and CspLB; both exponential and stationary growth phase), a cell surface antigen (CD4+ T – cell stimulator) (stationary growth phase only), superoxide dismutase (stationary growth phase only),

and the chemotaxis protein, CheY (exponential growth phase only). It is possible that the persistent strains employ specific proteins to adapt to alkaline challenge, while the sporadic strain induces a broader, potentially less effective, response, in an attempt to achieve homeostasis.

The cold – shock proteins are known to aid stress adaptation in *L. monocytogenes*, and have been associated with a range of stresses including cold, osmotic, antimicrobial, acidity and starvation (Schmid *et al.,* 2009; Gahan *et al.,* 1996). These proteins regulate transcription through a nucleic acid-dependant binding mechanism, binding to single stranded RNA, and acting as molecular chaperones by inhibiting the formation of secondary structures (Jones and Inouye, 2006). Expression of different subtypes of the cold – shock proteins (e.g. CspA, CspB, CspC and CspD) has been demonstrated to have a functional hierarchy, dependant on the particular stress (Schmid *et al.,* 2009). Furthermore subtypes of Csp proteins have been identified as essential for the transition to stationary growth phase (Graumann and Marahiel, 1999). In the current study, significantly increased abundance of CspLA and CspLB was identified in exponential phase, while only CspLA was significantly increased in stationary phase, relevant to the sporadic factory contaminant. CspA has been shown to increase in exponential growth phase, and decrease in stationary growth phase in *Escherichia coli*, while CspB is induced in the transition to stationary phase in *Bacillus subtilis* (Brandi *et al.,* 1999; Graumann and Marahiel, 1999). While Csp subtypes are certainly involved in both stress and the transition to stationary growth, based on reports studying these proteins, it appears that the same Csp subtype can fulfil different roles depending on the prevailing environmental conditions and the organism involved (Graumann and Marahiel, 1999; Jones and Inouye, 2006; Schmidt *et al.,* 2009; Yamanaka and Inouye, 1997). In the present study, it is possible that the observed increase in expression of CspLA and CspLB in exponential growth may provide protection during alkaline homeostasis in the persistent strains, with an increase in CspLB in the sporadic strain (levelling the abundances between strains) marking the transition to stationary growth phase, with CspLA continuing in a protective role in the persistent strains. Based on this notion, CspLB and CspLA could both have a role in alkaline homeostasis in *L. monocytogenes*, with the growth phase transitional role of CspLB superseding its contribution to alkaline homeostasis.

A similar response was observed with the chemotaxis response regulator CheY and lipoprotein TcsA (a lipoprotein that acts as a CD4+ T – cell stimulating

antigen). These proteins were significantly increased in the persistent strains in exponential growth phase and stationary phase respectively at pH8.5. CheY, coupled to flagellin, has a role in motility, virulence and cellular invasion, while TcsA is implicated in virulence (Dons *et al.*, 2004; Van – Schaik and Abee, 2005). The reasoning for increased expression relative to the sporadic strain at pH8.5 is unclear, possibly reflecting differences in serotype and/or serotype. Alternatively, it is possible that CheY and TcsA are constitutively expressed with other proteins that are directly associated with alkaline homeostasis, induced by a master regulator such as Positive Regulatory Factor A (PrfA) or σ^B.

The number of proteins identified that are associated with the cell envelope, transport and binding increased between exponential and stationary growth phase at pH7.3, with a decrease observed at pH8.5. However, the abundance of these proteins was increased at pH8.5 relative to pH7.3. A single protein was significantly increased in abundance at pH8.5 in the persistent *L. monocytogenes* strains in exponential growth phase at pH8.5. This was identified as lmo0171, a predicted cell surface / cell wall protein. BLASTP analysis (applied through the GenoList server) showed this protein was similar to the internalins of some *L. monocytogenes* strains (mainly lineage II). Therefore, this protein may be associated with virulence, although this remains speculative without further evidence.

In stationary growth phase at pH8.5, three cell envelope associated proteins, D – alanine—poly(phosphoribitol) ligase subunit 2 (DdlA), glucose – 1 – phosphate thymidylyltransferase (RfbA) and dTDP-4-dehydrorhamnose reductase (RfbD), and six transport and binding proteins (Crr, HPr, SufC, MetN, lmo2373 and OppA) were significantly increased in abundance in the persistent strains. DdlA is involved in the biosynthesis of D-alanyl-lipoteichoic acid, associated with cell wall biogenesis, while RfbA and RfbD are associated with nucleotide sugar metabolism, streptomycin biosynthesis, and polyketide sugar unit biosynthesis and the generation of a range of diverse products.

The ABC transporters identified are associated with the transport of carbohydrates, alcohols and acids, as well as oligopeptide binding protein A (OppA). The function of these proteins in alkaline homeostasis could be direct cytoplasmic acidification through importation of surrogate proton sources, however further work is required to confirm this. The role of OppA in stress tolerance is also associated with survival of the acidic pH experienced by *L. monocytogenes* following internalisation by macrophage, survival at elevated temperatures and osmotolerance

(Borezee *et al.*, 2000; Porteus, unpublished data). This may suggest a broad role for OppA in stress tolerance by *L. monocytogenes*. HPr, in contrast, is associated with regulation of the phosphotransferase system, and catabolite repression (Christensen *et al.*, 1999). Catabolite repression represents an important component of the global regulatory system in bacteria, associated with adaptation to an alternate carbon and energy source (Deutscher, 2008). As part of the adaptive alkaline shift, energy metabolism would be expected to adjust to accommodate the associated cellular processes. This has been reported before (Booth, 1985; Padan *et al.*, 2005; Hong and Brown, 2010) and supporting evidence was identified in the current study.

Significantly increased production of electron transport chain proteins, and proteins associated with glycolysis/gluconeogenesis were observed in the persistent *L. monocytogenes* strains in both exponential and stationary growth phase at pH8.5. It is possible that this increase in energy production may be directed at biogenesis of proteins associated with alkaline homeostasis. However, given the significant increase in the catabolite repression protein HPr, it is likely that an adaptive energy metabolic shift has been induced. This is evidenced by the significantly increased abundance of the regulator of sigma B – T (RsbT) protein and superoxide dismutase in the persistent strains at pH8.5. RsbT directly contributes to sigma B activation in *L. monocytogenes* during exposure to environmental and energy stresses, specifically, starvation, entry into stationary phase and reduced intracellular energy (ATP) levels (Boor and Chaturongakul, 2004). Superoxide dismutase is involved in the metabolism of reactive oxygen species, and as such, may indicate an increase of these compounds within the cell. This may reflect an increase in the reducing state of the cytoplasm, with subsequent electron leakage (thereby generating reactive oxygen species), and, consequent need to increase enzymes that metabolise reactive oxygen species. The increased reducing state may be caused through active acidification of the cytoplasm, or, alternatively, as part of an adaptive energy metabolic shift (Schafer and Buettner, 2001). The increase of superoxide dismutase in the persistent strains may reflect both an increased propensity, relative to the sporadic strain, to induce an adaptive metabolic shift, and an enhanced ability to counter the negative consequences of such a shift. Further work is required to confirm this hypothesised shift; however, it would be conducive to survival and persistence of *L. monocytogenes* within challenging environments.

To conclude, alkaline homeostasis in *L. monocytogenes* appears to involve a number of distinct, interdependent systems. Furthermore, these systems appear more

pronounced in stationary growth phase, suggesting overlap between the regulatory methods employed to counter alkaline and growth phase induced stress. The cells appear to respond to alkaline challenge through active acidification of the cytoplasm, stabilisation of regulatory elements, cytoplasmic buffering, increasing cell wall integrity and shifting energy metabolism. Many proteins appear to be involved in cytoplasmic buffering and channelling carbon into the production of acids. This is reflected both directly, as increased amino acid biosynthesis and substance transport for example, and indirectly, as redirection of various metabolic pathways and protein stabilisation to accommodate the protective response. Additionally, the response appears to include an energy metabolic shift, either as a consequence of, or to accommodate, the necessary physical adjustments for alkaline adaptation.

This study suggests the persistent *L. monocytogenes* strains investigated have an increased propensity to undergo an alkaline adaptation shift, and that the expression of virulence determinants could form part of the response, but this requires more analysis. Results from this work potentially suggest that a broad persistent phenotype exists that is better able to mobilise the cellular mechanisms conducive to withstanding pH flux. Furthermore, it seems possible that this response may have been imparted by the environment in which the strains have adapted. These strains could, at some stage, have been selected for colonization by being subjected to pH fluxes within the factory environments, and having available mechanisms to counter these environmental conditions. It is possible that the sporadic factory contaminants represent strains that have not been exposed to pH fluxes, and, as such, cannot rapidly mobilise these protective elements. Particularly concerning is the increased expression of virulence determinants in the persistent strains seemingly associated with the alkaline adaptive response. This could indicate a coupled virulence – survival response, induced by exposure to mild pH fluxes, which mimics the biological sequence of events that occur during passage through the alkaline portion of the mammalian gastrointestinal tract. The transition from environmental saprophyte to virulent pathogen may, in fact, form part of an innate response, that varies substantially at the strain-level. More work is required to better characterise the alkaline adaptation response in different *L. monocytogenes* strains, and this forms the basis for the following chapter.

CHAPTER 5
MEMBRANE ENRICHED PROTEIN ABUNDANCE PROFILE OF ALKALINE ADAPTED *LISTERIA MONOCYTOGENES* EGD-e

5.1 Introduction

The *Listeria monocytogenes* EGD-e genome encodes approximately 2846 proteins, and, to date, 1883 have been assigned a biological function, 258 remain to be assigned a biological function, and 705 are hypothetical (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi). These proteins are distributed throughout the cell cytoplasm, envelope and surface components of *L. monocytogenes* EGD-e, fulfilling distinct yet complementary roles. Proteins associated with the cell envelope are further distributed amongst the cell envelope compartmental layers (Figure 5.1). These proteins are of particular interest in ecophysiological studies, as they represent the interface between molecular mechanisms within the cell, and the extracellular environment.

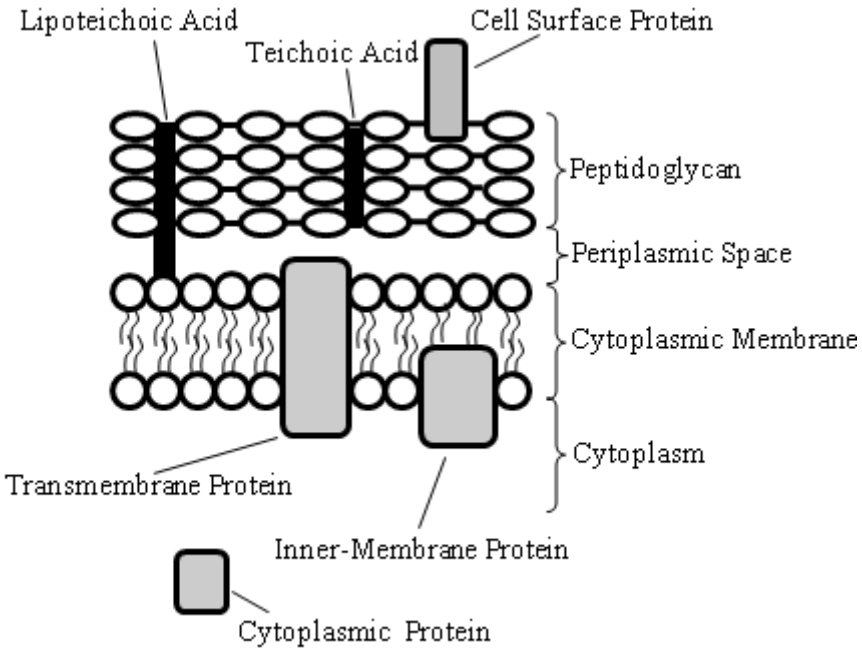


Figure 5.1 Simplified structure of the cell envelope of *Listeria monocytogenes*. Note: lipid anchored envelope proteins may also be present.

In this Chapter, membrane protein enriched extracts from an alkaline adapted *L. monocytogenes* strain cultured at 37°C were analysed using multidimensional protein identification technology (MuDPIT, described in Chapter 4). An alkaline extracellular environment interacts directly with the cell envelope and, as such, adaptive processes mediated through this compartment are of considerable interest. The cell membrane, in particular, represents the interface between the environment and the cytoplasm of the cell, thereby constituting the conduit through which all cellular processes directed at alkaline adaptive mechanisms must, to some extent, be mediated.

The cell envelope membrane component and associated macromolecules of *L. monocytogenes* have been associated with virulence, biofilm development and adaptation/resistance to environmental stress (Bierne and Cossart, 2007; Giotis *et al.*, 2007; Giotis *et al.*, 2008; Hunte *et al.*, 2005; Sokolovic *et al.*, 1993; Trémoulet *et al.*, 2002). Structurally, the cell membrane of *L. monocytogenes* is approximately 85 – 100 Angström (A) thick, and is composed of three layers; an inner and outer layer approximately 25 – 37A thick, and an inner layer approximately 20 – 25A thick (Ghosh and Carroll, 1968). The approximate composition of the *L. monocytogenes* membrane is 60% protein, 35% lipid, 12% nitrogen, 4% phosphorous, 2.3% carbohydrate (glucose, galactose, ribose, arabinose), 1.5% ribonucleic acid, 0.1% deoxyribonucleic acid, 0.4% amino sugar and 0.4% rhamnose. The lipid component can be further sub-categorised, comprising approximately 85% phospholipid, 20% neutral lipid, 3% phosphorous, 3% carbohydrate and 0.3% nitrogen (Ghosh and Carroll, 1968).

The roles that many membrane proteins fulfil under different environmental conditions remain to be definitively characterised. This is largely due to the difficulty in analysing these roles, as protein solubilisation is a key component of proteomics analysis, and many of the membrane proteins are highly insoluble. In particular, the response of *L. monocytogenes* membrane proteins under alkaline conditions, often experienced to varying extents in food processing environments due to the routine use of alkaline cleansing/sanitising agents, remains poorly described. So, too, are the interactions between the membrane and cell surface proteins, cytoplasmic proteins, and cell regulatory processes in general.

Under alkaline environmental conditions, it is known that the cell wall of *L. monocytogenes* undergoes modifications, with increased transporter activity,

altered surface structures and phenotypic adjustment (Giotis *et al.,* 2007a; Giotis *et al.,* 2008). These adjustments are coupled to cytoplasmic modifications, directed primarily at cellular maintenance and reduction of cytoplasmic alkalisation (Giotis *et al.,* 2008). *L. monocytogenes* is commonly found asymptomatically in the mammalian gut but also can be a pathogen invading *via* the intestine. As such it is capable of surviving passage through the variable pH of the gastrointestinal tract (pH2 – pH10), and these processes are suspected to represent a normal response occurring at 37°C (Sleator *et al.,* 2009). Information on the proteins driving the alkaline adaptation response in *L. monocytogenes* at 37°C could provide insight into the mechanisms supporting survival, persistence and pathogenesis within the mammalian intestine, and could, in part, explain the basis for the organism's ability to develop resistance to alkaline cleansing and sanitising agents (Kastbjerg *et al.,* 2009). Furthermore, it could provide information for the identification of novel targets that may be exploited to control this pathogen in food processing environments.

With this in mind, this study aimed to build on previous work (Chapter 4; Giotis *et al.,* 2008) investigating alkaline adaption in *L. monocytogenes.* Enriched membrane protein abundance profiles of cell fractions from *Listeria monocytogenes* strain EGD-e cultured at 37°C in late-exponential growth phase at pH 7.3 and adapted to pH 9.0 were analysed using MuDPIT (Delahunty and Yates, 2005). A higher pH was used in an attempt to induce a greater alkaline tolerance response based on knowledge attained from Chapter 4. Specifically, cell protein extracts were compared to (i) define the association between proteins from all cellular compartments with the alkaline adaptation response observed in *L. monocytogenes* strain EGD-e at 37°C, (ii) assess membrane protein enriched expression profiling as an indirect indicator of the alkaline adaptation responses associated with the cell wall, and, (iii) assess the effect on spectral quantitation by normalisation of spectral counts against an internal standard of known concentration.

5.2 Materials and Methods

5.2.1 Bacterial culture

L. monocytogenes strain EGD-e was recovered from frozen storage (Appendix 1.1) and grown in 10 mL of brain – heart infusion broth (CM225, 'BHI';

OXOID, Australia; Appendix 2.2) incubated at 37 °C for twenty hours. This was repeated in fresh BHI. Four × 10 mL of fresh BHI broth was prepared and the pH level of two of these was adjusted to 9.0 (±0.1) through addition of 4 M NaOH (Sigma-Aldrich, Castle Hill, Australia). After autoclaving, the pH of both media (two × pH 7.3, and two × pH 9.0) was confirmed using an Orion 250A pH meter (Orion Research Inc, USA), and further adjusted using sterile NaOH and HCl if required. The initial culture conditions were repeated over a period of 7 days by transferring (daily) a 100 µL aliquot of the cultures to 9.9 mL of fresh BHI broth (with pH adjusted accordingly) to acclimatise the cultures to the growth conditions. Finally, two replicate (2 × biological replicates)10 mL cultures of each pH condition were prepared, incubated at 37°C, and harvested at exponential phase ($OD_{600} \approx 0.6 \pm 0.1$) for MudPIT analysis.

5.2.2 Preparation of bacterial protein extracts for MuDPIT analysis

The bacterial cultures were centrifuged at 14000 × g for 5 minutes in an Eppendorf 51417 centrifuge and the supernatant was discarded. The cell pellets were gently resuspended (washed) in phosphate buffered saline (Appendix 1.6), transferred to 1.5 mL Eppendorf Protein LoBind microcentrifuge tubes (Sigma-Aldrich, Castle Hill, Australia), and centrifuged again at 14000 × g for 5 minutes. The wash was repeated three times. Following the final wash, the supernatant was discarded and the cell pellet frozen in liquid nitrogen. The pellet was thawed on ice for 15 minutes. A ProteoPrep membrane extraction kit (Sigma-Aldrich, Castle Hill, Australia) was used to prepare enriched membrane protein extracts (membrane and cytosolic proteins) according to manufacturer instructions.

The approximate concentration of the protein extracts was determined by standard Bradford assay (Sigma-Aldrich, Castle Hill, Australia) in a 96-well microplate format (Greiner Scientific, Australia) using a 2mg/mL Bovine Serum Albumin (BSA) standard (Sigma-Aldrich, Castle Hill, Australia). Absorbance at 595nm was measured using a Benchmark microplate reader (BioRad, USA). An internal standard consisting of 1.25 µg/mL of the BSA standard (Appendix 1.4) was added to the enriched membrane protein extracts and they were divided into separate fractions according to the schema in Table 5.1. Finally, the concentrations of the fractions were adjusted to ≈50 µg/mL, and they were subjected to trypsin digestion as described in Chapter 4.

Table 5.1 Sample fractionation of the biological replicates prior to trypsin digestion.

Treatment	Biological Replicate	Fraction
pH7.3	1	1
		2
		3
	2	1
		2
		3
pH9.0	1	1
		2
		3
	2	1
		2
		3

5.2.3 MuDPIT analysis

MuDPIT analysis was performed as described in Chapter 4. Protein identifications were assigned as described in Chapter 4 with the following adjustments. The tandem mass spectra data were only compared against the *L. monocytogenes* EGD-e (2846 putative protein encoding genes) National Centre for Biotechnology Institute protein database. An identification integration analysis was applied to the TPP output. This enabled the level of false positive peptide and protein identifications to be estimated and controlled. As *L. monocytogenes* EGD-e is well characterised, increased stringency was applied to the analysis. Identifications with a Peptide Prophet score of <0.9, protein group probabilities of <0.9, and protein identifications with a Protein Prophet score of <0.9 were not considered for further analysis. Single unique peptide identifications were validated as described in Chapter 4. Finally, a decoy database search was conducted with the same integration analysis filtering criteria as the actual search (<0.9).

5.2.4 Functional grouping and biological role assignment

Functional grouping of protein identifications was performed manually using the J. Craig Venter Institute Comprehensive Microbial Resource (JCVI-CMR) (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntlm01) *L. monocytogenes* EDG-e primary annotation summary database. Biological role assignment of protein

identifications was performed manually using the "Genolist" *L. monocytogenes* serovar 1/2a EGD-e database (Version 3) (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList.woa/wa/goToTaxoRank?level=Listeria%20monocytogenes%20EGD-e), and Kyoto Encyclopedia of Genes and Genomes (KEGG) *L. monocytogenes* serovar 1/2a EGD-e database (http://www.genome.jp/kegg/).

5.2.5 Differential protein abundance

Relative protein abundances were determined by the spectral counting method (Wang *et al.*, 2003) using TPP Xpress quantitation software (Version 2.1) in conjunction with X!Tandem analysis. Spectra counts for each protein assignment were normalised against the BSA internal standard using the formula:

$$\text{Normalised}_{\text{BSA}} = [(nf_1+\sigma) + (nf_2+\sigma) + (nf_3+\sigma)] / [(bsaf_1) + (bsaf_2) + (bsaf_3)]$$

Where nf_n is the spectral count of a given protein assignment in each fraction, σ is a pseudo spectral count of 0.5 assigned to account for spectral counts of 0 in any given fraction, and $bsaf_n$ is the BSA spectra count for each fraction. Correlation between biological replicates was assessed by linear regression using Microsoft Excel® 2007 (Microsoft Inc., U.S.A.).

Spectral count ratios (fold change; R_{SC}) were determined according to the method described by Old *et al.*, (2005):

$$R_{SC} = \log_2\left[\frac{(n_2+f)}{(n_1+f)}\right] + \log_2\left[\frac{(t_1-n_1+f)}{(t_2-n_2+f)}\right]$$

Where n_1 and n_2 are the spectral counts for proteins 1 and 2 respectively, t_1 and t_2 are the total spectral counts for samples $n_1(t_1)$ and $n_2(t_2)$, and f is a correction factor of 1.25 predetermined to be optimal by Old *et al.*, (2005).

Significantly different protein abundance was assessed, independent of R_{SC} determination, by the spectral index – permutation analysis (SpI) method recently described by Fu *et al.* (2008). This method accounts for protein absence in some samples, minimising statistical bias (Fu *et al.*, 2008). Significance was assigned at $p \leq 0.05$. A *G*-test was also performed on the data, according to the method outlined in Chapter 4, for comparison with the spectral index method. Data grouping, assembly,

statistical analysis and refinement were performed using Microsoft Excel® 2007 (Microsoft Inc., U.S.A.).

5.3 Results

A total of 534 individual proteins were identified following integration analysis. These were assigned to 18 main and 96 sub-functional roles based on the JCVI-CMR functional ontology system. No protein identifications were assigned based on the decoy database search. A summary of the proteins and spectral counts for the main functional groups is presented in Table 5.2. Each main functional group of this system, with associated sub-functional groups, is described in the following sections. A complete dataset is provided in Appendix 5.2.

Table 5.2 Summary table detailing the number of proteins identified in *L. monocytogenes* membrane-enriched samples, and the pooled spectra counts*, for each protein functional category as defined by the JCVI – CMR functional ontology system.

Functional Category	# Proteins	Spectral* Count pH7.3	Spectral Count* pH9.0
Amino Acid Biosynthesis	11	198	154
Biosynthesis of Cofactors, Prosthetic Groups / Carriers	15	72	25
Cell Envelope	22	94	68
Cellular Processes	26	2593	1411
Central Intermediary Metabolism	13	100	79
DNA Metabolism	14	58	38
Energy Metabolism	51	1295	955
Fatty Acid and Phospholipid Metabolism	14	89	67
Mobile and Extrachromosomal Element Functions	1	6	5
Protein Fate	31	426	779
Protein Synthesis	72	1466	1885
Purines, Pyrimidines, Nucleosides and Nucleotides	18	131	120
Regulatory Functions	28	69	105
Transcription	15	84	74
Transport and Binding Proteins	26	526	505
Viral Functions	2	17	6
Function Unknown	120	1479	2309
Multiple Roles	55	501	564

*Spectral counts have been normalised to account for sampling depth.

High correlation was observed between biological replicates for the spectral counts of individual protein assignments, of each treatment, following normalisation against the BSA internal standard (Figure 5.2). The indices generated from the spectral counts corresponded to a normal distribution (acceptable level of kurtosis), satisfying the primary assumption for SpI – permutation analysis, and were therefore

suitable for valid statistical comparison (Fu *et al.,* 2008; Figure 5.3). The complete SpI – permutation statistical output is presented in Appendix 5.3.

Analysis by *G*-test highlighted greater numbers of significantly different spectral abundances ($G > 3.841$; χ^2, 1df; $p<0.05$) than SpI, with 408 (76.4%) of proteins differing significantly from the pH7.3 treatment. SpI analysis accounts for missing data values between samples/fractions, while the *G*-test does not, and adjusts the statistical threshold accordingly resulting in a more conservative statistical analysis (Fu *et al.,* 2008). The SpI method was selected for this study to ensure maximum stringency, while minimising bias that may be introduced by the absence of protein identifications in some samples/fractions. The fold change determination (ratio spectral count, Rsc) employed does not account for missing values between samples/fractions and because of this, the statistically significant SpI may, in some instances, appear incongruent to the expected fold change. With this in mind, results must be considered separately as fold change (Rsc) and SpI. The fold change results compares the total differences in the spectral abundances observed between pH treatments, while the SpI highlights proteins that differed very significantly (and were present in every fraction analysed) between pH treatments. All fold change values that were greater than those identified as significant by SpI were significant by *G*-test, however they contained missing values in some fractions, which negatively affected the SpI analysis. To ensure a conservative statistical analysis, significant *G*-values, although presented, were not considered in the interpretation of results in this study.

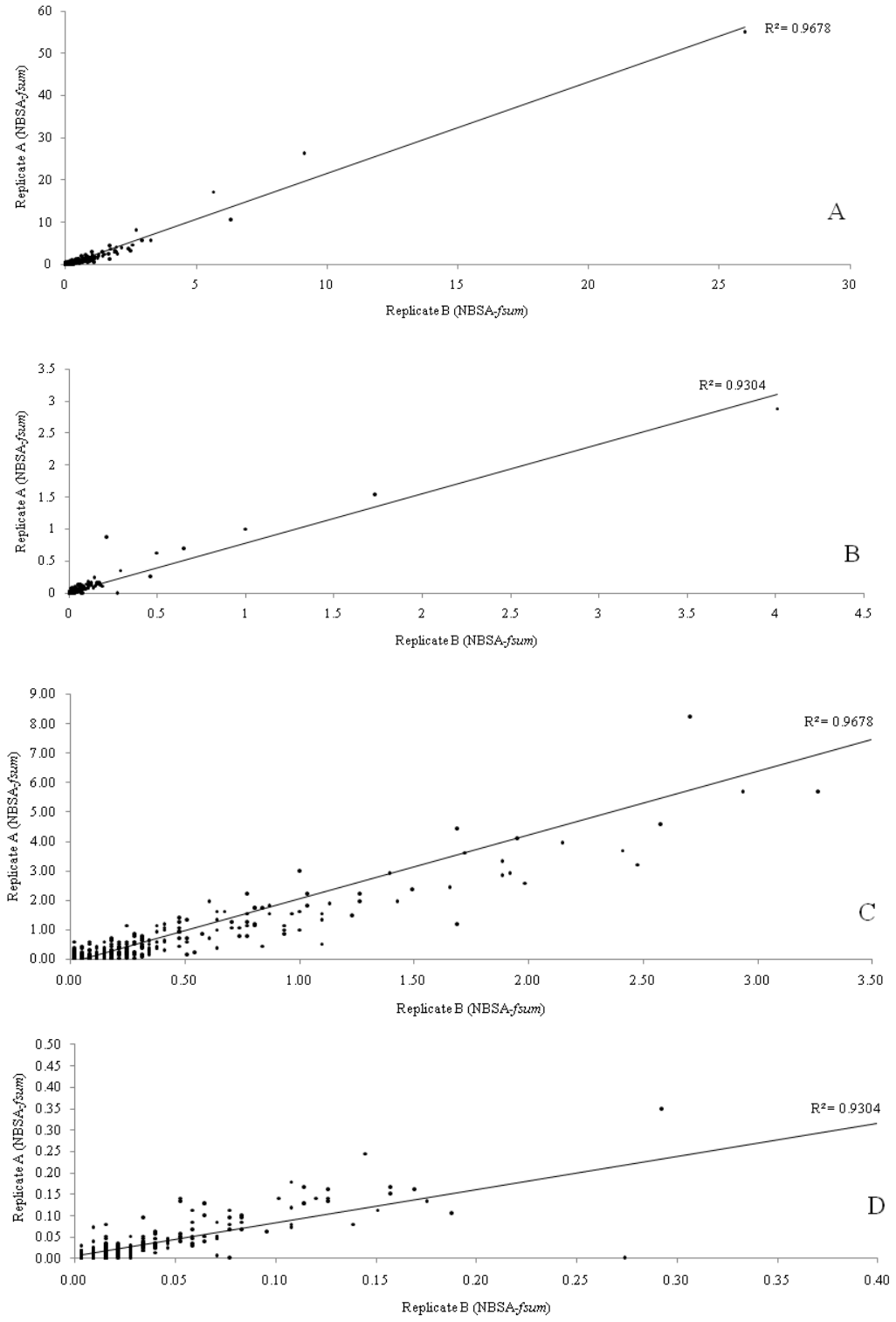


Figure 5.2 Correlation between biological replicates (n = 2) of the pooled spectra count (NBSA-*fsum*) of three fractions for each pH treatment replicate. Individual protein spectral counts (n = 535) were first normalised against the spectral count of an internal standard (1.25µg/mL BSA). Linear regression values of 0.9678 and 0.9304 were obtained for the pH 9.0 (A and C) and pH7.3 (B and D) treatments respectively. Figures C (pH9.0) and D (pH7.3) highlight the lowest NBSA-*fsum* regions in Figures 5.2A (C) and 5.2B (D).

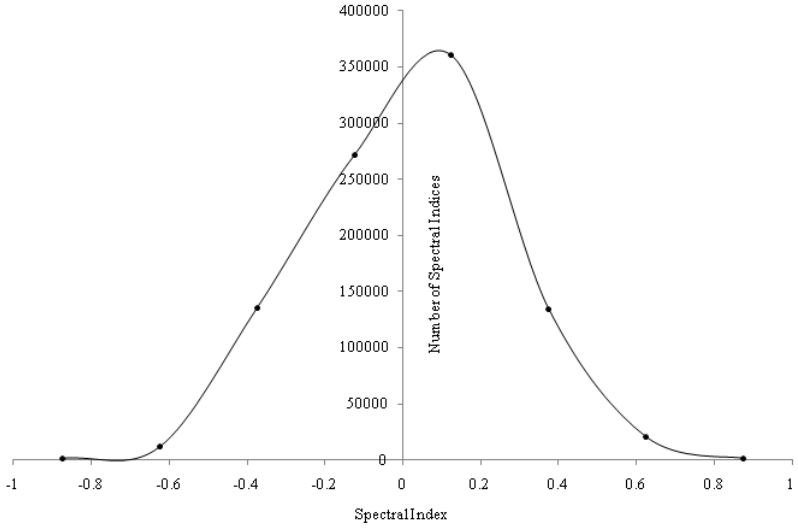


Figure 5.3 The distribution of spectral indices derived from the individual protein spectral count data, calculated and presented, according to the method described by Fu *et al.,* (2008).

5.3.1 Proteins associated with amino acid biosynthesis

Eleven proteins (2.06% of the total protein identifications) were directly associated with biosynthesis of amino acids (Table 5.3). Increased abundance was observed for six of these when compared with the control treatment. Those proteins predominantly function in aromatic amino acid biosynthesis, while decreased abundance was predominantly associated with proteins involved in aspartate biosynthesis. No significant difference in abundance was identified for this group (SpI<±0.5; p>0.05).

Table 5.3 Protein identifications associated with amino acid biosynthesis. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment (p<0.05). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo1435	0.820	*		Aspartate-Family	Diaminopimelate and lysine biosynthesis
lmo1984	0.429	*		Pyruvate Family	Biosynthesis of valine, leucine and isoleucine
lmo0491	0.290			Aromatic Amino Acid	Biosynthesis of phenylalanine, tyrosine and tryptophan
lmo1600	0.290			Aromatic Amino Acid	Biosynthesis of phenylalanine, tyrosine and tryptophan
lmo1631	0.290			Aromatic Amino Acid	Biosynthesis of phenylalanine, tyrosine and tryptophan
lmo0978	0.230			Pyruvate Family	Biosynthesis of valine, leucine and isoleucine
lmo1299	-1.377	*		Glutatmate-Family	Metabolism of alanine, aspartate and glutamate
lmo0223	-1.397	*		Serine Family	Metabolism of sulphur, cysteine and methionine
lmo1437	-1.477	*		Aspartate-Family	Biosynthesis of lysine, methionine and threonine
lmo1011	-2.630	*		Aspartate-Family	Biosynthesis of lysine
lmo1907	-2.630	*		Aspartate-Family	Diaminopimelate and lysine biosynthesis

5.3.2 Proteins associated with biosynthesis of cofactors, prosthetic groups and carriers

Fifteen of the identified proteins (2.80% of the total protein identifications) were associated with biosynthesis of cofactors, prosthetic groups and carriers (Table 5.4). Abundance was predominantly decreased relative to the control treatment, with only four proteins showing increased abundance when cultured at pH9.0. Significantly reduced abundance of lmo2101 was observed (SpI≥0.5; p<0.05).

Table 5.4 Protein identifications associated with biosynthesis of cofactors, prosthetic groups and carriers. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment (p<0.05). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo1554	1.207	*		Haeme, Porphyrin and Cobalamin	Porphorin and chlorophyl metabolism
lmo1093	0.359			Pyridine Nucleotides	NH(3)-dependent NAD(+) synthetase
lmo2211	0.290			Haeme, Porphyrin and Cobalamin	Porphorin and chlorophyl metabolism
lmo1592	0.290			Thiamine	Biosynthesis of thiamine
lmo2256	-0.093			Thiamine	Peptidase
lmo1673	-0.172			Menaquinone and Ubiquinone	Ubiquinone and other terpenoid-quinone biosynthesis
lmo0968	-1.251	*		Pyridine Nucleotides	NAD metabolism
lmo1873	-1.407	*		Folate	Folate biosynthesis
lmo0662	-1.477	*		Thiamine	Biosynthesis of pyridoxal phosphate
lmo2101	***-1.904***	*****	*****	***Pyridoxine***	***Biosynthesis of pyridoxal phosphate***
lmo1317	-1.938	*		Other	Secondary metabolite/terpenoid backbone biosynthesis
lmo1902	-2.088	*		Pantothenate and Coenzyme A	Magnesium binding (proton acceptor), pantothenate biosynthesis
lmo0225	-2.325	*		Folate	Folate biosynthesis
lmo0236	-2.325	*		Other	Secondary metabolite/terpenoid backbone biosynthesis
lmo1360	-2.882	*		Folate	Folate biosynthesis

5.3.3 Proteins associated with the cell envelope

Twenty two of the identified proteins (4.11% of the total protein identifications) are associated with the cell envelope (Table 5.5). Increased abundance of proteins associated with peptidoglycan-based cell wall biogenesis, regulation of cell shape and flavin mononucleotide (FMN) binding was evident. Proteins involved with peptidoglycan-based cell wall biogenesis and regulation were also found to have decreased abundance, although to a lesser extent. Significantly decreased abundance of lmo0972 protein, associated with cell wall organisation, was observed (SpI≥0.5; p<0.05).

5.3.4 Proteins associated with cellular processes

Proteins associated with cellular processes constituted 4.86% of the total protein identifications (n = 26) (Table 5.6). These included proteins functioning in adaptation to atypical conditions, cell division, detoxification and pathogenesis. Increased abundance of proteins associated with cellular redox homeostasis, cell division, pathogenesis and FMN binding was evident. Significantly increased abundance of cell division proteins (inhibitors), pathogenesis elements, proteolysis factors, post-translational molecular chaperones, and proteins functioning in regulation of cell wall biosynthesis were observed (SpI≥0.5; p<0.05).

Interestingly, while increased abundance of flagellar basal body protein was detected, decreased abundance of extracellular flagellin filament was detected. Other proteins observed in decreased abundance included those associated with superoxide metabolism, stress induced transcriptional regulation, and surface associated proteins such as adhesins (P60 protein) and the pathogenesis factors lmo0202 and lmo0433.

Table 5.5 Protein identifications associated with the cell envelope. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo0198	1.207	*		Biosynthesis and Degradation of Surface Polysaccharides and Lipopolysaccharides	Magnesium ion binding, lipopolysaccharide biosynthesis,peptidoglycan based cell wall biogenesis: regulation of cell shape
lmo2638	1.066	*		Other	Flavin mononucleotide binding
lmo1521	0.820	*		Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Peptidoglycan catabolism
lmo2035	0.820	*		Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Peptidoglycan based cell wall biogenesis: regulation of cell shape, UDP-N-acetylgalactosamine biosynthetic process
lmo2036	0.820	*		Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Peptidoglycan based cell wall biogenesis: regulation of cell shape, folate biosynthesis
lmo2525	0.385			Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	ATP binding, cell morphogenesis
lmo1892	0.359			Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Peptidoglycan based cell wall biogenesis: regulation of cell shape
lmo0441	0.290			Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Penicillin binding/Peptidoglycan based cell wall biogenesis
lmo2229	0.290			Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Penicillin binding/Peptidoglycan based cell wall biogenesis/peptidase activity
lmo2754	0.290			Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Proteolysis
lmo0880	0.290			Other	Cell surface adhesion protein
lmo0130	-0.149			Other	Nucleotide catabolism
lmo2039	-0.559	*		Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Penicillin binding/Peptidoglycan based cell wall biogenesis
lmo1547	-0.946	*		Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Regulation of cell shape
lmo1078	-1.330	*		Biosynthesis and Degradation of Surface Polysaccharides and Lipopolysaccharides	UDP glucose metabolism
lmo1829	-1.407	*		Surface Structures	Cell surface adhesion protein
lmo2537	-1.407	*		Biosynthesis and Degradation of Surface Polysaccharides and Lipopolysaccharides	UDP-N-acetylgalactosamine biosynthetic process, lipopolysaccharide biosynthesis
lmo1226	-1.407	*		Other	Integral membrane protein, ATP binding function
lmo1527	-1.407	*		Other	Intracellular protein transport/secretion across membrane, type II secretion system
lmo2038	-1.938	*		Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan	Peptidoglycan based cell wall biogenesis: regulation of cell shape, folate biosynthesis
lmo2052	-2.325	*		Biosynthesis and Degradation of Surface Polysaccharides and Lipopolysaccharides	Coenzyme A biosynthesis
lmo0972	***-4.349***	*****	*****	***Biosynthesis and Degradation of Murein Sacculus and Peptidoglycan***	***Cell wall organisation, biogenesis, pathogenesis***

Table 5.6 Protein identifications associated with cellular processes. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo2217	***4.270***	*****	*****	***Adaptations to Atypical Conditions***	***Unknown***
lmo0204	***2.736***	*****	*****	***Pathogenesis***	***Integral to membrane, virulence related***
lmo1601	***2.200***	*****	*****	***Adaptations to Atypical Conditions***	***Stress adaptation controlled by Sigma B***
lmo0220	***1.764***	*****	*****	***Cell Division***	***Protein catabolism/proteolysis***
lmo1879	***1.584***	*****	*****	***Adaptations to Atypical Conditions***	***Transcriptional regulation induced by stress***
lmo2033	***1.207***	*****	*****	***Cell Division***	***Inhibits cell division***
lmo1583	1.083	*		Detoxification	Cellular redox homeostasis
lmo0433	0.820	*		Pathogenesis	Cell wall, pathogenesis
lmo1580	0.754	*		Adaptations to Atypical Conditions	Stress adaptation controlled by Sigma B
lmo2020	***0.636***	*****	*****	***Cell Division***	***Unknown, likely involved in initiation and inhibition of cell division***
lmo0197	***0.310***	*****	*****	***Adaptations to Atypical Conditions***	***Capsular polysaccharide production***
lmo1804	0.290			Cell Division	Chromosome organisation and biogenesis
lmo2506	0.290			Cell Division	Integral membrane protein, involved in potassium transport and cell division
lmo0713	0.290			Chemotaxis and Motility	Flagellar motility, part of basal body
lmo2235	0.290			Detoxification	FAD/FMN binding/oxidation- reduction
lmo1967	0.259			Toxin Production and Resistance	Unknown function
lmo1888	-0.473			Cell Division	Cell division/regulation of cell shape
lmo0202	-0.819	*		Pathogenesis	Cell surface- pathogenesis
lmo2790	-1.089	*		Cell Division	DNA binding partition protein
lmo0394	-1.407	*		Pathogenesis	Cell surface protein P60, adhesion, pathogenesis
lmo1439	-1.486	*		Detoxification	Oxidation/reduction, superoxide metabolism
lmo2016	-1.582	*		Adaptations to Atypical Conditions	Transcriptional regulation induced by stress
lmo1364	-1.779	*		Adaptations to Atypical Conditions	Transcriptional regulation induced by stress
lmo0433	-1.938	*		Pathogenesis	Cell surface-pathogenesis
lmo2032	-2.248	*		Cell Division	Cell division, barrier septum formation
lmo0690	-4.263	*		Chemotaxis and Motility	Flagellin motility, extracellular flagellin-based flagellum filament

5.3.5 Proteins associated with central intermediary metabolism

Thirteen proteins (constituting 2.43% of the total protein identifications) were found to be associated with central intermediary metabolism (Table 5.7). Again, a protein associated with FMN binding (lmo2638) was present in increased abundance, and was significantly increased relative to the control treatment (SpI≥0.5; $p<0.05$). Other proteins present in increased amounts included those involved with oxidoreduction, metal ion binding, carbohydrate metabolism (glycolysis/gluconeogenesis) and amino-sugar (glutamine) metabolism.

Decreased protein abundance relative to the control predominated in this group. Proteins detected at lower levels included those functioning in starch metabolism, FAD binding and a number of hydrolases.

Table 5.7 Protein identifications associated with central intermediary metabolism. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo1737	0.820	*		Other	Oxidoreductase activity, metal ion binding
lmo2638	***0.732***	*****	*****	***Other***	***FAD binding, electron carrier activity, oxidative phosphorylation***
lmo0536	0.155			Other	Carbohydrate metabolism-gluconeogenesis/glycolysis
lmo0727	0.054			Amino Sugars	Glutamine metabolism, carbohydrate biosynthesis
lmo0370	-0.307			Phosphorous Compounds	Unknown function, similar to phosphonoacetate hydrolase
lmo2577	-0.559			Other	Hydrolase activity
lmo0018	-0.960	*		Other	Carbohydrate metabolism-starch/sucrose
lmo0663	-1.407	*		Other	Hydrolase activity
lmo0956	-1.938	*		Amino Sugars	N-acetylglucosamine metabolism
lmo1351	-1.938	*		Sulphur Metabolism	Unknown, similar to a rhodanese related sulphurtransferase
lmo2389	-1.938	*		Other	FAD binding, electron carrier activity,
lmo0271	-2.630	*		Other	Carbohydrate metabolism-starch/sucrose
lmo2700	-3.448	*		Other	Oxidoreductase activity, metal ion binding

5.3.6 Proteins associated with DNA metabolism

Proteins associated with DNA metabolism constituted 2.62% (n = 14) of the total protein identifications (Table 5.8). Twelve of the 14 proteins functioned in DNA replication, recombination and repair, with the remaining two proteins involved in degradation of DNA.

Significantly increased abundance was observed for lmo0001 protein (SpI≥0.5; $p<0.05$), which is involved in the initiation and regulation of DNA

replication. Only three other proteins within this group were present in increased abundance relative to the control treatment, and the increase was minor. These are involved in DNA repair, topological change/underwinding and DNA catabolism. Most of the proteins identified within this group showed decreased abundance and, as with those with increased abundance, functioned in DNA repair, topological change/underwinding, mismatch repair, DNA replication and the SOS response.

Table 5.8 Protein identifications associated with DNA metabolism. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo0001	***1.512***	*****	*****	***DNA Replication, Recombination and Repair***	***Initiation and regulation of DNA replication***
lmo2267	0.385			Degradation of DNA	DNA repair
lmo1286	0.290			DNA Replication, Recombination and Repair	DNA topological change, underwinding
lmo1362	0.290			Degradation of DNA	DNA catabolism
lmo0006	-0.028			DNA Replication, Recombination and Repair	DNA topological change, underwinding
lmo2308	-0.307			DNA Replication, Recombination and Repair	DNA replication and repair
lmo1403	-0.559			DNA Replication, Recombination and Repair	Mismatch repair
lmo1398	-0.559			DNA Replication, Recombination and Repair	DNA recombination, repair, SOS response
lmo0002	-0.960	*		DNA Replication, Recombination and Repair	DNA replication
lmo0045	-0.960	*		DNA Replication, Recombination and Repair	DNA replication and repair
lmo2488	-1.089	*		DNA Replication, Recombination and Repair	SOS response, nucleotide excision repair
lmo0007	-1.251	*		DNA Replication, Recombination and Repair	DNA topological change, underwinding
lmo1758	-1.407	*		DNA Replication, Recombination and Repair	DNA replication and repair
lmo1404	-1.938	*		DNA Replication, Recombination and Repair	Mismatch repair

5.3.7 Proteins associated with energy metabolism

Fifty-one of the proteins identified (constituting 9.53% of the total protein identifications) are associated with energy metabolism (Table 5.9). More than half of these proteins (n = 34) decreased in abundance relative to the control treatment. Notably, five proteins associated with ATP-Proton motive force interconversion decreased in abundance. These are integral membrane proteins that function in hydrogen ion transport. Proteins associated with NAD binding, electron transport, cell redox homeostasis and a number of glycolytic elements also decreased in abundance.

Significantly increased abundance of proteins coupled to intramolecular transferase activity, the pentose phosphate shunt, electron transport and cation binding was detected (SpI≥0.5; $p<0.05$). Increased abundance of oxidoreductase, and phosphorylase was also evident.

5.3.8 Proteins associated with fatty acid and phospholipid metabolism

Proteins with fatty acid and phospholipid metabolic roles constituted 2.62% (n = 14) of the total protein identifications (Table 5.10). All of these proteins were directly associated with the biosynthesis and degradation of fatty acids and phospholipids.

Significantly increased abundance of lmo2089 and lmo1381 was detected (SpI≥0.5; $p<0.05$). These proteins have fatty acid and phospholipid degradation (metabolic hydrolase) and unknown (acylphosphatase) activity respectively. All of the other proteins with increased abundance identified within this group were associated with fatty acid and phospholipid biosynthesis, however, the abundance of some identified proteins associated with these functions decreased. Also present in decreased amounts were proteins functioning in FAD binding and lipid A biosynthesis.

5.3.9 Proteins associated with mobile and extrachromosomal element functions

A single protein associated with mobile and extrachromosomal element functions was identified (lmo0114), despite there being 23 genes involved with these functions confirmed on the *Listeria monocytogenes* EGD-e genome to date. Lmo0114 has prophage functions, has homology to repressor C1 from lactococcal bacteriophage, and was abundant relative to the control treatment.

Table 5.9 Protein identifications associated with energy metabolism. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo2475	***3.144***	*****	*****	***Sugars***	***Intramolecular transferase activity, phosphotransferase, mg ion binding***
lmo0517	***3.078***	*****	*****	***Glycolysis/Gluconeogenesis***	***Unknown, likely a glycolysis associated phosphoglyceromutase***
lmo0184	***1.685***	*****	*****	***Sugars***	***Carbohydrate metabolism, cation binding***
lmo1305	***0.834***	*****	*****	***Pentose Phosphate pathway***	***Transketolase, pentose phosphate pathway***
lmo1936	0.820	*		Other	Glycerol-3-phosphate metabolism, oxidation/reduction, phospholipid biosynthesis
lmo2487	***0.729***	*****	*****	***Electron Transport***	***Unknown function, possible regulatory role***
lmo1072	0.665	*		Glycolysis/Gluconeogenesis	Pyruvate carboxylase, gluconeogenesis
lmo2743	0.664	*		Pentose Phosphate pathway	Pentose phosphate shunt
lmo0183	0.664	*		Sugars	Carbohydrate metabolism
lmo1570	0.600	*		Glycolysis/Gluconeogenesis	Phosphorylation, glycolysis
lmo1634	0.538			Fermentation	Alcohol metabolism/carbon utilisation
lmo0319	0.504			Sugars	Carbohydrate metabolism, beta-glucosidase, cation binding
lmo0830	0.290			Glycolysis/Gluconeogenesis	Unknown function
lmo1538	0.290			Other	Glycerol-3-phosphate metabolism, oxidation/reduction
lmo1053	0.237			Pyruvate Dehydrogenase	Glycolysis/gluconeogenesis
lmo2457	0.171			Glycolysis/Gluconeogenesis	Glycolysis/gluconeogenesis, pentose phosphate shunt
lmo1052	0.064			Pyruvate Dehydrogenase	Oxidoreductase
lmo2436	-0.040			Sugars	Regulation of transcription
lmo0268	-0.055			Glycolysis/Gluconeogenesis	Unknown function
lmo2094	-0.172			Sugars	Metal ion binding
lmo1571	-0.240			Glycolysis/Gluconeogenesis	Phophofructokinase, glycolysis
lmo1992	-0.254			Fermentation	Unknown function
lmo1373	-0.254			Pyruvate Dehydrogenase	Unknown function
lmo2528	-0.425			ATP Proton Motive Force Interconversion	Integral membrane protein, hydrogen ion transporter
lmo2455	-0.556			Glycolysis/Gluconeogenesis	Cell surface protein, glycolysis and pathogenesis
lmo2425	-0.559			Amino Acids and Amines	Glycine decarboxylation via the glycine cleavage system

lmo0182	-0.559		Sugars	Carbohydrate metabolism
lmo1254	-0.559		TCA Cycle	Trehalose catabolism
lmo0943	-0.559		Fermentation	Cellular iron ion homeostasis/stress response
lmo2532	-0.559		ATP Proton Motive Force Interconversion	Integral membrane protein, hydrogen ion transporter
lmo2459	-0.726	*	Glycolysis/Gluconeogenesis	NAD binding, oxidation reduction, sugar metabolism
lmo1579	-0.728	*	Amino Acids and Amines	Electron carrier activity
lmo1293	-0.864	*	Other	Glycerol-3-phosphate metabolism, oxidation/reduction
lmo0092	-0.936	*	ATP Proton Motive Force Interconversion	Integral membrane protein, hydrogen ion transporter
lmo2456	-1.194	*	Glycolysis/Gluconeogenesis	Glycolysis
lmo0907	-1.251	*	Glycolysis/Gluconeogenesis	Unknown, likely a glycolysis associated phosphoglyceromutase
lmo2367	-1.351	*	Glycolysis/Gluconeogenesis	Glycolysis/gluconeogenesis
lmo0261	-1.407	*	Sugars	Carbohydrate metabolism, cation binding, hydrolysing O-glycosyl compounds
lmo2477	-1.407	*	Sugars	Galactose metabolism
lmo2556	-1.450	*	Glycolysis/Gluconeogenesis	Glycolysis
lmo1813	-1.517	*	Amino Acids and Amines	Gluconeogenesis
lmo1371	-1.614	*	Pyruvate Dehydrogenase	Cell redox homeostasis, oxidation reduction
lmo0088	-1.731	*	ATP Proton Motive Force Interconversion	Integral membrane protein, hydrogen ion transporter
lmo2564	-1.848	*	Other	Aromatic compound catabolism
lmo1376	-1.863	*	Pentose Phosphate pathway	NAD binding, oxidation reduction, sugar metabolism
lmo1619	-1.938	*	Amino Acids and Amines	D-amino acid biosynthesis and catabolism
lmo2110	-1.938	*	Sugars	Carbohydrate metabolism,mannsoe -6-phosphate isomerase, zinc ion binding
lmo1233	-2.034	*	Electron Transport	Electron transport, cell redox homeostasis
lmo1566	-2.034	*	TCA Cycle	TCA cycle, isocitrate dehydrogenase (NADP+) activity
lmo2530	-2.248	*	ATP Proton Motive Force Interconversion	Integral membrane protein, hydrogen ion transporter
lmo2205	-5.747	*	Glycolysis/Gluconeogenesis	Glycolysis

Table 5.10 Protein identifications associated with fatty acid and phospholipid metabolism. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo0885	1.512	*		Biosynthesis	Fatty acid biosynthesis
lmo1357	1.512	*		Biosynthesis	Biotin binding acetylCoA carboxylase
lmo2089	***1.073***	*****	*****	***Degradation***	***Hydrolase activity, metabolism***
lmo1381	***0.778***	*****	*****	***Other***	***Unknown function, acylphosphatase activity***
lmo1807	0.712	*		Biosynthesis	Fatty acid biosynthesis
lmo1946	0.290			Degradation	Unknown function
lmo1808	-0.172			Biosynthesis	Fatty acid biosynthesis
lmo2201	-0.394			Biosynthesis	Fatty acid biosynthesis
lmo1396	-0.559			Biosynthesis	Integral membrane protein, phospholipid biosynthesis
lmo0786	-1.542	*		Other	FAD/FMN binding/oxidation- reduction
lmo2524	-1.938	*		Biosynthesis	Fatty acid, lipid A biosynthesis
lmo0970	-2.069	*		Biosynthesis	Oxidoreductase activity
lmo1806	-2.325	*		Biosynthesis	Fatty acid biosynthesis
lmo2202	-3.096	*		Biosynthesis	Fatty acid biosynthesis

5.3.10 Proteins associated with protein fate

Thirty-one proteins associated with protein fate were identified, constituting 5.79% of the total protein identifications (Table 5.11). Significantly increased abundance of metalloendopeptidase, and proteins associated with stress induced protein folding and metabolism of reactive oxygen species was detected (SpI≥0.5; $p<0.05$). Also in increased abundance were proteins involved with: protein transmembrane transport, RNA binding, N-terminal acetylation, protein folding and refolding, and protein, peptide, and glycopeptide degradation functions.

A significant decrease in abundance was detected for a serine-type endopeptidase (lmo2468), associated with protein, peptide, glycopeptide degradation (SpI≥0.5; $p<0.05$). A stress chaperone, (lmo2206), cell surface trafficking element, peptide deformylase, protein dimerisation, and various proteolytic proteins were also detected in decreased abundance relative to the control treatment.

Table 5.11 Protein identifications associated with protein fate. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo2069	***3.453***	*****	*****	***Protein Folding and Stabilisation***	***Protein folding, response to stress***
lmo1474	***2.613***	*****	*****	***Protein Folding and Stabilisation***	***Protein folding, response to stress***
lmo2510	1.130	*		Protein and Peptide secretion and Trafficking	Protein transport across the membrane
lmo1407	***0.852***	*****	*****	***Protein Modification and Repair***	***Reactive oxygen species metabolism***
lmo0216	0.820	*		Protein Folding and Stabilisation	RNA binding
lmo1709	***0.693***	*****	*****	***Protein Modification and Repair***	***Proteolysis***
lmo2376	0.664	*		Protein Folding and Stabilisation	Accelerates protein folding
lmo1493	***0.664***	*****	*****	***Degradation of Proteins, Peptides and Glycopeptides***	***Metalloendopeptidase, proteolysis***
lmo1473	0.553			Protein Folding and Stabilisation	Protein folding, response to stress
lmo0203	0.290			Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo0961	0.290			Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo2068	0.193			Protein Folding and Stabilisation	Protein refolding
lmo1529	0.125			Protein and Peptide secretion and Trafficking	Unknown function
lmo1603	-0.002			Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo1268	-0.172			Degradation of Proteins, Peptides and Glycopeptides	Protein transport
lmo1780	-0.559			Degradation of Proteins, Peptides and Glycopeptides	Peptide metabolism, protein dimerisation
lmo0387	-0.559			Degradation of Proteins, Peptides and Glycopeptides	Unknown function
lmo1620	-0.970	*		Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo1051	-1.089	*		Protein Modification and Repair	Peptide deformylase, translation
lmo0963	-1.089	*		Protein Folding and Stabilisation	Integral membrane protein, proteolysis
lmo1611	-1.225	*		Degradation of Proteins, Peptides and Glycopeptides	Unknown function
lmo0842	-1.407	*		Protein and Peptide secretion and Trafficking	Cell surface protein
lmo1711	-1.407	*		Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo1578	-1.904	*		Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo1279	-1.938	*		Protein Folding and Stabilisation	Protease activity
lmo2188	-2.248	*		Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo2468	***-2.435***	*****		***Degradation of Proteins, Peptides and Glycopeptides***	***Serine type endopeptidase, proteolysis***
lmo1217	-2.589	*		Degradation of Proteins, Peptides and Glycopeptides	Unknown function
lmo1354	-2.600	*		Degradation of Proteins, Peptides and Glycopeptides	Proteolysis
lmo0292	-2.630	*		Degradation of Proteins, Peptides and Glycopeptides	Serine type endopeptidase, proteolysis
lmo2206	-3.272	*		Degradation of Proteins, Peptides and Glycopeptides	Stress chaperone

5.3.11 Proteins associated with protein synthesis

A large proportion (13.46%, n = 72) of the proteins identified were directly associated with protein synthesis (Table 5.12). The role of many of these proteins (n = 43) was translation, including 17 that were present in significantly increased amounts relative to the control treatment (SpI≥0.5; $p<0.05$). Two proteins that significantly increased in abundance were able to be assigned a role beyond translation alone. These were lmo1314, involved in the termination of translation, and lmo1756, involved in the regulation of translation fidelity (glutamine hydrolase). Increased abundances were also detected for a number of aminoacetylase's,

including arginyl, cysteinyl, seryl, prolyl and asparaginyl aminoacetylase's. Interestingly, decreased aminoacetylase abundances were also detected for amino acid substrates other than those with increased abundance. These included phenylalanyl, methionyl, leucyl, lysyl, glutamyl, isoleucyl, valyl and alanyl aminoacetylases.

A single protein (lmo2596) was significantly decreased in abundance relative to the control treatment (SpI≥0.5; $p<0.05$). This protein has a translational role. Other proteins identified to have decreased abundance within this group included those associated with signal transduction, translation elongation and initiation, and queosine biosynthesis.

5.3.12 Proteins associated with purines, pyrimidines, nucleosides and nucleotides

Proteins associated with purines, pyrimidines, nucleosides and nucleotides constituted 3.36% (n = 18) of the total protein identifications (Table 5.13). Considerable overlap between biological roles of proteins that were present in increased and decreased abundance was noted. Distinct biological functions were attributed to five proteins, all present in increased amounts. These included a protein associated with cytidine metabolism, a ligase involved in the formation of carbon-nitrogen bonds, a protein associated with deoxy-thymidine monophosphate biosynthesis, oxidation-reduction and tRNA modification proteins. The latter two proteins were present in significantly increased abundance (SpI≥0.5; $p<0.05$), along with a protein associated with purine nucleoside metabolism (lmo1856).

Table 5.12 Protein identifications associated with protein synthesis. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo1797	***4.594***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo1542	***4.497***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo0053	***3.230***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo0249	***3.144***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo1596	***3.144***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2618	***3.061***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo1314	***2.736***	*****	*****	***Other***	***Translational termination***
lmo2623	***2.614***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2511	***2.268***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo0044	***1.874***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2633	1.803	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo2616	***1.765***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo0250	***1.621***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo1756	***1.517***	*****	*****	***tRNA Aminoacylation***	***Glutamine hydrolysing enzyme, regulation of translation fidelity***
lmo2597	***1.458***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2630	***1.312***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2613	***1.265***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2624	***1.265***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2617	***1.017***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2632	0.989	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo2627	***0.910***	*****	*****	***Ribosomal Proteins: Synthesis and Modification***	***Translation***
lmo2655	0.847	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo1458	0.820	*		tRNA Aminoacylation	Arginyl--tRNA aminoacetylation
lmo2614	0.820	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo2610	0.423			Other	Translational initiation
lmo1540	0.407			Ribosomal Proteins: Synthesis and Modification	Translation

lmo2628	0.290			Ribosomal Proteins: Synthesis and Modification	Translation
lmo0239	0.290			tRNA Aminoacylation	Cysteinyl-tRNA aminoacetylation
lmo2747	0.290			tRNA Aminoacylation	Seryl-tRNA aminoacetylation
lmo2620	0.259			Ribosomal Proteins: Synthesis and Modification	Translation
lmo0251	0.201			Ribosomal Proteins: Synthesis and Modification	Translation
lmo2629	0.193			Ribosomal Proteins: Synthesis and Modification	Translation
lmo1330	0.154			Ribosomal Proteins: Synthesis and Modification	Translation
lmo2654	0.122			Other	Translational elongation
lmo1319	0.094			tRNA Aminoacylation	Prolyl--tRNA aminoacetylation
lmo1896	0.075			tRNA Aminoacylation	Asparaginyl-tRNA aminoacetylation
lmo1787	0.015			Ribosomal Proteins: Synthesis and Modification	Translation
lmo2608	-0.035			Ribosomal Proteins: Synthesis and Modification	Translation
lmo1785	-0.059			Other	Translational initiation
lmo0248	-0.093			Ribosomal Proteins: Synthesis and Modification	Translation
lmo0211	-0.105			Ribosomal Proteins: Synthesis and Modification	Translation/general stress
lmo2626	-0.150			Ribosomal Proteins: Synthesis and Modification	Translation
lmo1519	-0.172			tRNA Aminoacylation	Aspartyl--tRNA aminoacetylation
lmo1938	-0.184			Ribosomal Proteins: Synthesis and Modification	Translation
lmo1754	-0.254			tRNA Aminoacylation	Glutamine hydrolysing enzyme, translation
lmo2615	-0.290			Ribosomal Proteins: Synthesis and Modification	Translation
lmo2548	-0.353			Ribosomal Proteins: Synthesis and Modification	Translation
lmo2596	***-0.508***	*****	*****	**Ribosomal Proteins: Synthesis and Modification**	**Translation**
lmo0046	-0.559	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo1658	-0.596	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo2631	-0.658	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo2811	-0.864	*		Translation Factors	Signal transduction, tRNA modification
lmo1755	-0.946	*		tRNA Aminoacylation	Glutamine hydrolysing enzyme, translation
lmo1657	-1.377	*		Other	Translational elongation
lmo1222	-1.407	*		tRNA Aminoacylation	Phenylalanyl-tRNA aminoacetylation, tRNA processing
lmo2622	-1.407	*		Ribosomal Proteins: Synthesis and Modification	Translation
lmo1325	-1.407	*		Other	Translation initiation

lmo0177	-1.634	*	tRNA Aminoacylation	Methionyl-tRNA aminoacetylation
lmo2653	-1.762	*	Other	Translational elongation
lmo1660	-1.782	*	tRNA Aminoacylation	Leucyl--tRNA aminoacetylation
lmo2561	-1.904	*	tRNA Aminoacylation	Arginyl--tRNA aminoacetylation
lmo2605	-1.938	*	Ribosomal Proteins: Synthesis and Modification	Translation
lmo2625	-1.938	*	Ribosomal Proteins: Synthesis and Modification	Translation
lmo1531	-1.938	*	Translation Factors	Queosine biosynthesis
lmo2607	-2.034	*	Ribosomal Proteins: Synthesis and Modification	Translation
lmo0228	-2.069	*	tRNA Aminoacylation	Lysyl-tRNA aminoacetylation
lmo0237	-2.248	*	tRNA Aminoacylation	Glutamyl-tRNA aminoacetylation
lmo2019	-2.435	*	tRNA Aminoacylation	Isoleucyl-tRNA aminoacetylation
lmo1552	-2.630	*	tRNA Aminoacylation	Valyl--tRNA aminoacetylation
lmo2619	-2.882	*	Ribosomal Proteins: Synthesis and Modification	Translation
lmo1355	-3.096	*	Other	Translational elongation
lmo1504	-3.967	*	tRNA Aminoacylation	Alanyl--tRNA aminoacetylation

Table 5.13 Protein identifications associated with purines, pyrimidines, nucleosides and nucleotides. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo0219	***2.165***	*****	*****	***Salvage of Nucleotides and Nucleosides***	***Purine ribonucleotide salvage, tRNA modification, translation***
lmo2155	***1.512***	*****	*****	***2'-Deoxyribonucleotide Metabolism***	***DNA replication, oxidation-reduction***
lmo1874	1.207	*		2'-Deoxyribonucleotide Metabolism	dTMP biosynthesis
lmo1856	***0.821***	*****	*****	***Salvage of Nucleotides and Nucleosides***	***Purine nucleoside metabolism***
lmo1954	0.290			Salvage of Nucleotides and Nucleosides	Deoxyribonucleotide catabolism, metabolic compound salvage
lmo1771	0.290			Purine Ribonucleotide Biosynthesis	Unknown function, ligase involved in the formation of carbon-nitrogen bonds
lmo1835	0.290			Pyrimidine Ribonucleotide Biosynthesis	Pyrimidine nucleotide biosynthesis, arginine biosynthesis
lmo1463	0.290			Salvage of Nucleotides and Nucleosides	Cytidine metabolism
lmo0055	0.212			Purine Ribonucleotide Biosynthesis	Purine nucleotide biosynthesis
lmo2758	0.072			Purine Ribonucleotide Biosynthesis	GMP biosynthesis
lmo1096	-0.059			Purine Ribonucleotide Biosynthesis	GMP biosynthesis
lmo2538	-0.446			Salvage of Nucleotides and Nucleosides	Nucleoside metabolism, uracil salvage
lmo0199	-0.559			Purine Ribonucleotide Biosynthesis	Nucleoside metabolism, ribonucleoside monophosphate biosynthesis
lmo1993	-0.773	*		Salvage of Nucleotides and Nucleosides	Pyrimdine nucleoside metabolism
lmo1773	-1.407	*		Purine Ribonucleotide Biosynthesis	Purine ribonucleotide biosynthesis
lmo2559	-1.904	*		Pyrimidine Ribonucleotide Biosynthesis	Pyrimidine nucleotide biosynthesis, glutamine metabolism
lmo1497	-2.630	*		Salvage of Nucleotides and Nucleosides	Pyrimidine salvage
lmo2611	-3.096	*		Nucleotide and Nucleoside Interconversions	Nucleotide biosynthesis

5.3.13 Proteins associated with regulatory functions

Fifteen proteins (2.80% of the total protein identifications) have regulatory functions (Table 5.14). Significantly increased abundance of RNA processing and degradation proteins (lmo1327 and lmo0218 respectively) was detected (SpI≥0.5; $p<0.05$). Similarly, significantly decreased abundance of a DNA dependant RNA polymerase was observed (SpI≥0.5; $p<0.05$). Other proteins identified in increased abundance were associated both with the degradation of RNA and regulation of transcription (elongation regulator). Decreased abundance of proteins functioning in DNA dependant transcriptional regulation, mRNA catabolism and transcription termination was noted.

5.3.14 Proteins associated with transcription

Twenty-eight proteins (5.23% of the total protein identifications) were directly associated with transcription (Table 5.14). Four of these were significantly more abundant than in the control treatment (SpI≥0.5; $p<0.05$). These included a protein associated with catabolite control, one of unknown function, a DNA dependent, negative regulator of transcription, and a sugar-hydrogen integral membrane symporter. A number of other proteins that function in DNA dependent transcription regulation were also identified in increased amounts, along with two component signal transduction elements.

Proteins associated with two component signal transduction were also present in decreased quantities, along with proteins associated with manganese ion homeostasis, amino acid phosphorylation and arginine biosynthesis.

5.3.15 Transport and binding proteins

Twenty-six proteins (constituting 4.86% of the total protein identifications) associated with transport and binding were identified (Table 5.15). Within this group, 15 transporters were observed, including three with significantly increased abundance relative to the control treatment (SpI≥0.5; $p<0.05$). These included a peptide transporter, an unknown transporter and a sugar-hydrogen symporter. Five sugar-hydrogen symporters were detected overall, all with increased abundance relative to the control treatment. Other transporters with increased abundance

included ion transporters (copper, cobalt and mercury), carbohydrate, alcohol and acid transporters, and amino acid transporters. An oxo-donor oxidoreductase protein was identified in increased amounts (lmo1327).

A number of proteins within this group had decreased abundance relative to the control treatment. Many of these were also transporters, and a number were associated with the cell surface and outer membrane.

5.3.16 Proteins with viral functions

Two proteins associated with viral functions were identified (lmo2072 and lmo2219); despite there being 49 such genes confirmed on the *Listeria monocytogenes* EGD-e genome to date. These were observed in decreased and increased amounts respectively relative to the control treatment. The first, lmo2072, is similar to putative DNA binding protein, while lmo2219 is similar to a post-translocation molecular chaperone.

Table 5.14 Protein identifications associated with transcription and regulatory functions. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count.*Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo1599	***3.144***	*****	*****	***Other***	***DNA dependant regulation of transcription, catabolite control***
lmo1280	***2.613***	*****	*****	***Other***	***DNA dependant negative regulation of transcription***
lmo0888	***2.330***	*****	*****	***Other***	***Unknown function***
lmo0427	***1.696***	*****	*****	***Other***	***Integral to membrane, sugar-hydrogen symporter activity, phosphoenol-pyruvate dependant phosphotransferase system***
lmo1298	0.981	*		Other	DNA dependant regulation of transcription
lmo0797	0.820	*		DNA Interactions	DNA dependant regulation of transcription
lmo0168	0.820	*		Other	Unknown function
lmo0443	0.820	*		Other	Unknown function
lmo2792	0.664	*		DNA Interactions	Unknown function, DNA sequence specific DNA binding
lmo2421	0.564			Other	Peptidyl-histidine phosphorylation, two component signal transduction (phosphorelay)
lmo1060	0.290			Other	Transcription regulation, two component signal transduction (phosphorelay)
lmo1262	0.290			Other	Unknown function, DNA sequence specific DNA binding
lmo1850	0.290			Other	DNA dependant regulation of transcription
lmo2337	0.290			Other	DNA dependant regulation of transcription
lmo2728	0.290			Other	DNA dependant regulation of transcription
lmo2493	0.007			Other	DNA dependant regulation of transcription
lmo1956	-0.559			Other	DNA dependant regulation of transcription
lmo1878	-0.559			Other	Manganese ion homeostasis
lmo1721	-1.089	*		Other	Integral membrane protein, transcription regulation, two component signal transduction (phosphorelay)
lmo0288	-1.407	*		Other	Integral membrane protein, transcription regulation, two component signal transduction (phosphorelay)
lmo0535	-1.407	*		Other	DNA dependant regulation of transcription
lmo1820	-1.407	*		Other	Protein amino acid phosphorylation
lmo2004	-1.407	*		Other	DNA dependant regulation of transcription
lmo0785	-1.477	*		Other	Integral membrane protein, phosphoenol-pyruvate sugar dependant phosphotransferase system
lmo1377	-1.477	*		Other	Transcription regulation, two component signal transduction (phosphorelay)
lmo1367	-1.938	*		Other	DNA dependant regulation of transcription, arginine biosynthesis
lmo2593	-1.938	*		Other	DNA dependant regulation of transcription

lmo1948	-3.283	*		Other	Transcription regulation, two component signal transduction (phosphorelay)
lmo1327	***2.684***	*****	*****	***RNA Processing***	***rRNA processing***
lmo0218	***1.209***	*****	*****	***Degradation of RNA***	***Unknown function, RNA binding transferase***
lmo0844	0.820	*		Degradation of RNA	Unknown function
lmo1496	0.664	*		Transcription Factors	Transcription elongation regulator
lmo0891	0.290			Transcription Factors	Unknown function, ATP binding
lmo0258	***-0.283***		*****	***DNA Dependant RNA Polymerase***	***Response to antibiotics, DNA dependant regulation of transcription***
lmo2560	-0.559			DNA Dependant RNA Polymerase	DNA dependant regulation of transcription
lmo1793	-0.559			RNA Processing	rRNA processing
lmo2191	-0.559			Other	DNA dependant regulation of transcription
lmo2606	-1.251	*		DNA Dependant RNA Polymerase	DNA dependant regulation of transcription
lmo1454	-1.938	*		Transcription Factors	DNA dependant regulation of transcription initiation
lmo0894	-2.034	*		Transcription Factors	Negative regulation of transcription, DNA dependant protein amino acid phosphorylation
lmo1331	-2.248	*		Degradation of RNA	RNA processing, mRNA catabolism
lmo0246	-2.248	*		Transcription Factors	DNA dependant regulation of transcription, transcription antitermination
lmo1322	-2.882	*		Transcription Factors	Regulation of transcription termination

Table 5.15 Protein identifications associated with transport and binding. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count. *Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo0507	***1.764***	*****	*****	***Carbohydrates, Alcohols and Acids***	***Integral to membrane, sugar-hydrogen symporter activity, phosphoenol-pyruvate dependant phosphotransferase system***
lmo0181	***1.598***	*****	*****	***Unknown Substrate***	***Unknown, transporter***
lmo2193	***1.130***	*****	*****	***Amino Acids, Peptides and Amines***	***Peptide transport***
lmo1389	***0.970***	*****	*****	***Carbohydrates, Alcohols and Acids***	***Unknown function, ATP binding***
lmo2195	0.820	*		Amino Acids, Peptides and Amines	Integral membrane transporter
lmo1003	0.431			Carbohydrates, Alcohols and Acids	Integral to membrane, sugar-hydrogen symporter activity, phosphoenol-pyruvate dependant phosphotransferase system
lmo2362	0.291			Amino Acids, Peptides and Amines	Transmembrane amino acid transport
lmo1372	0.290			Amino Acids, Peptides and Amines	Aldehyde/oxo donor oxidoreductase, metabolism
lmo0859	0.290			Carbohydrates, Alcohols and Acids	Transporter
lmo1017	0.290			Carbohydrates, Alcohols and Acids	Integral to membrane, sugar-hydrogen symporter activity, phosphoenol-pyruvate dependant phosphotransferase system
lmo1852	0.290			Cations and Iron Carrying Compounds	Copper/mercury ion transmembrane transport
lmo2600	0.290			Unknown Substrate	Cobalt ion transmembrane transport
lmo1002	0.018			Carbohydrates, Alcohols and Acids	Integral to membrane, sugar-hydrogen symporter activity, phosphoenol-pyruvate dependant phosphotransferase system
lmo2396	-0.028			Amino Acids, Peptides and Amines	Cell surface protein, protein binding
lmo0278	-0.724	*		Carbohydrates, Alcohols and Acids	ATP binding cassette, transmembrane substance transport
lmo0135	-0.869	*		Amino Acids, Peptides and Amines	Transport
lmo0810	-1.407	*		Amino Acids, Peptides and Amines	Outer membrane bound, transport
lmo0559	-1.407	*		Unknown Substrate	Metal ion transmembrane transport
lmo0925	-1.407	*		Unknown Substrate	Transmembrane movement of substances
lmo2007	-1.407	*		Unknown Substrate	Transport
lmo2415	-1.407	*		Unknown Substrate	Oligopeptide transport
lmo1014	-1.477	*		Amino Acids, Peptides and Amines	Membrane protein, amino acid transport
lmo1636	-1.938	*		Unknown Substrate	Unknown function, ATP binding
lmo2192	-2.325	*		Amino Acids, Peptides and Amines	Peptide transport
lmo2114	-2.882	*		Unknown Substrate	Unknown function, ATP binding
lmo2372	-2.882	*		Unknown Substrate	Unknown function, ATP binding

5.3.17 Proteins of unknown function

The greatest proportion (22.43%, n = 120) of the total protein identifications were assigned as being of unknown function (Table 5.16). Many have been assigned putative functions that likely correspond to functional roles already detailed, however these remain to be definitively characterised.

Table 5.16 Protein identifications with unknown and hypothetical functions. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count. *Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo2703	***2.849***	*****	*****	***Unknown General***	***Unknown***
lmo0392	2.165	*		Unknown General	Integral membrane protein
lmo2692	***2.165***	*****	*****	***Unknown General***	***Unknown***
lmo2569	1.978	*		Unknown General	Unknown
lmo1388	***1.819***	*****	*****	***Unknown General***	***Binds to lipids***
lmo1028	***1.687***	*****	*****	***Unknown General***	***Unknown***
lmo2853	***1.673***	*****	*****	***Unknown General***	***Unknown***
lmo1668	***1.569***	*****	*****	***Unknown General***	***Unknown***
lmo0962	***1.378***	*****	*****	***Unknown General***	***LemA epitope***
lmo1001	***1.241***	*****	*****	***Unknown General***	***Unknown***
lmo0100	1.207	*		Unknown General	Unknown
lmo0900	1.207	*		Unknown General	Unknown
lmo1180	1.207	*		Unknown General	Unknown
lmo1643	1.207	*		Unknown General	Unknown
lmo1649	***1.207***	*****	*****	***Unknown General***	***Unknown***
lmo2120	***1.207***	*****	*****	***Unknown General***	***Unknown***
lmo1826	1.083	*		Unknown General	DNA dependant transcription
lmo1934	1.081	*		Unknown General	Unknown
lmo2223	***1.057***	*****	*****	***Unknown General***	***Unknown***
lmo0974	0.951	*		Unknown General	Techoic acid biosynthesis, pathogenesis
lmo0955	0.916	*		Unknown General	Unknown
lmo1229	***0.829***	*****	*****	***Unknown General***	***Unknown***
lmo1067	0.820	*		Unknown General	GTPase
lmo2216	0.820	*		Unknown General	Catalyst
lmo0558	0.820	*		Unknown General	Unknown
lmo1324	0.820	*		Unknown General	Unknown
lmo1468	0.820	*		Unknown General	Unknown
lmo1602	0.820	*		Unknown General	Unknown
lmo1663	0.820	*		Unknown General	Unknown
lmo1757	0.820	*		Unknown General	Unknown
lmo1763	0.820	*		Unknown General	Unknown
lmo1861	0.820	*		Unknown General	Unknown
lmo1998	0.820	*		Unknown General	Unknown
lmo2030	0.820	*		Unknown General	Unknown
lmo2340	0.820	*		Unknown General	Unknown
lmo1612	***0.652***	*****		***Unknown General***	***Unknown***
lmo2707	0.565			Unknown General	Unknown
lmo1489	0.564			Unknown General	Unknown

lmo0355	0.367		Unknown General	Electron carrier activity
lmo0673	0.359		Unknown General	Unknown
lmo1338	0.359		Unknown General	Unknown
lmo0273	0.290		Enzyme of Unknown Specificity	N-acetyltransferase
lmo0163	0.290		Unknown General	Unknown
lmo0322	0.290		Unknown General	Unknown
lmo0484	0.290		Unknown General	Antibiotic biosynthesis, oxidation reduction
lmo0702	0.290		Unknown General	Unknown
lmo0719	0.290		Unknown General	Unknown
lmo0814	0.290		Unknown General	Unknown
lmo1075	0.290		Unknown General	Unknown
lmo1159	0.290		Unknown General	Unknown
lmo1291	0.290		Unknown General	Unknown
lmo1606	0.290		Unknown General	Unknown
lmo1656	0.290		Unknown General	Unknown
lmo1862	0.290		Unknown General	Unknown
lmo0775	0.259		Unknown General	Unknown
lmo1692	0.259		Unknown General	Unknown
lmo2414	0.259		Unknown General	Unknown
lmo0302	-0.028		Unknown General	Unknown
lmo0553	-0.028		Unknown General	Unknown
lmo2214	-0.028		Unknown General	Unknown
lmo1059	-0.040		Unknown General	Unknown
lmo1814	-0.172		Unknown General	Unknown
lmo2474	-0.172		Unknown General	Unknown
lmo1718	-0.246		Unknown General	Unknown
lmo1213	-0.559		Unknown General	Unknown
lmo1323	-0.559		Unknown General	Unknown
lmo1336	-0.559		Unknown General	Unknown
lmo2118	-0.559		Unknown General	Calcium/magnesium ion binding, carbohydrate metabolism
lmo1257	-0.559		Unknown General	Unknown
lmo2785	-0.559		Unknown General	Hydrogen peroxide catabolism
lmo0391	-0.746	*	Unknown General	Unknown
lmo0579	-0.946	*	Unknown General	Unknown
lmo0774	-1.089	*	Unknown General	Unknown
lmo1499	-1.089	*	Unknown General	Unknown
lmo1791	-1.194	*	Unknown General	Unknown
lmo2248	-1.330	*	Unknown General	Unknown
lmo1491	-1.407	*	Unknown General	GTP binding
lmo0437	-1.407	*	Unknown General	Regulation of nitrogen utilisation, transcription termination activity
lmo0530	-1.407	*	Unknown General	Unknown
lmo0600	-1.407	*	Unknown General	Unknown
lmo1236	-1.407	*	Unknown General	Unknown
lmo1321	-1.407	*	Unknown General	Unknown
lmo1501	-1.407	*	Unknown General	Unknown
lmo1794	-1.407	*	Unknown General	Unknown
lmo1866	-1.407	*	Unknown General	Unknown
lmo1918	-1.407	*	Unknown General	Unknown
lmo1922	-1.407	*	Unknown General	Unknown
lmo2029	-1.407	*	Unknown General	Unknown
lmo2221	-1.407	*	Unknown General	Unknown
lmo2391	-1.407	*	Unknown General	Unknown
lmo2473	-1.407	*	Unknown General	Unknown
lmo1515	-1.477	*	Unknown General	DNA binding, iron ion binding
lmo1283	-1.477	*	Unknown General	Unknown

lmo2491	-1.477	*		Unknown General	Unknown
lmo1240	-1.782	*		Enzyme of Unknown Specificity	Hydrolase
lmo0903	-1.782	*		Unknown General	Unknown
lmo0004	-1.938	*		Unknown General	RNA binding
lmo0429	-1.938	*		Unknown General	Mannose metabolism
lmo0791	-1.938	*		Unknown General	Unknown
lmo0796	-1.938	*		Unknown General	Unknown
lmo1541	-1.938	*		Unknown General	Unknown
lmo1750	-1.938	*		Unknown General	Unknown
lmo2031	-1.938	*		Unknown General	Unknown
lmo2051	-1.938	*		Unknown General	Unknown
lmo2258	-1.938	*		Unknown General	Unknown
lmo2828	-1.938	*		Unknown General	Unknown
lmo0479	-2.034	*		Unknown General	Putative secreted protein
lmo2041	-2.325	*		Unknown General	Methyltransferase
lmo2411	-2.325	*		Unknown General	Unknown
lmo1594	-2.435	*		Unknown General	Integral membrane protein, septal ring formation
lmo1399	***-2.630***	*****		***Unknown General***	***2',3'-cyclic-nucleotide 2'-phosphodiesterase activity; RNA binding***
lmo1528	-2.630	*		Unknown General	Unknown
lmo2113	-2.630	*		Unknown General	Unknown
lmo2406	-2.630	*		Unknown General	Unknown
lmo2220	-2.882	*		Unknown General	3'-5'-exoribonuclease activity; nucleic acid binding
lmo2417	-2.883	*		Unknown General	Transporter (MetQ)
lmo0930	-3.283	*		Unknown General	Unknown
lmo1249	-3.283	*		Unknown General	Unknown
lmo1503	***-3.967***	*****	*****	***Unknown General***	***Unknown***
lmo2709	-4.581	*		Unknown General	Unknown

5.3.18 Proteins with multiple roles

Fifty-five (10.28% of the total identifications) *L. monocytogenes* EGD-e proteins characterised by the JCVI-CMR functional ontology system were assigned to multiple biological functions/roles (Table 5.17). These proteins had similar biological functions to many that have been described in the previous sections, and therefore, although they are involved in a range of biological processes, are likely fulfilling similar purposes.

Significantly increased abundance of four proteins associated with the phosphoenol-pyruvate dependant phosphotransferase system were identified, for example. In addition, proteins associated with folding and transport of proteins, DNA repair, proteolysis, sugar metabolism and translation were present in increased abundances. These are probably associated with a more specific JCVI-CMR functional assignment given the experimental treatment, and based on other proteins

identified in this study. Similar results were observed for proteins detected in decreased abundance within this group.

Table 5.17 Protein identifications associated with multiple biological functions. Those designated as unknown, means that the *specific* biological function is not yet known. Grey shaded cells highlight significantly different protein abundances as determined by SpI. **Ratio spectral count. *Significantly different spectral abundance relative to the pH7.3 treatment ($p<0.05$). *G* = *G*-test; SpI = spectral index.

Protein	Rsc**	*G*	SpI	Sub-Role	Biological Function
lmo1719	***2.613***	*****	*****	***Multiple Roles***	***Integral to membrane, phosphoenol-pyruvate dependant phosphotransferase system***
lmo1647	***2.330***	*****	*****	***Multiple Roles***	***Unknown***
lmo2335	***2.165***	*****	*****	***Multiple Roles***	***Integral to membrane, phosphoenol-pyruvate dependant phosphotransferase system***
lmo2683	***2.072***	*****	*****	***Multiple Roles***	***Integral to membrane, phosphoenol-pyruvate dependant phosphotransferase system***
lmo1434	***1.888***	*****	*****	***Multiple Roles***	***Hydrolase***
lmo0027	***1.679***	*****	*****	***Multiple Roles***	***Unknown***
lmo1349	***1.512***	*****	*****	***Multiple Roles***	***Glycine decarboxylation***
lmo1671	***1.512***	*****	*****	***Multiple Roles***	***Unknown***
lmo2685	***1.319***	*****	*****	***Multiple Roles***	***Integral to membrane, phosphoenol-pyruvate dependant phosphotransferase system***
lmo0201	1.207	*		Multiple Roles	Intracellular signaling, lipid catabolism
lmo2696	***1.159***	*****	*****	***Multiple Roles***	***Glycerol metabolism***
lmo2369	***0.856***	*****	*****	***Multiple Roles***	***Translation***
lmo2539	0.820	*		Multiple Roles	Serine/glycine metabolism
lmo1295	0.820	*		Multiple Roles	Unknown
lmo1285	0.820	*		Multiple Roles	Unknown
lmo2097	0.820	*		Multiple Roles	Unknown
lmo2373	0.820	*		Multiple Roles	Unknown
lmo2599	0.820	*		Multiple Roles	Unknown
lmo1027	0.820	*		Multiple Roles	Unknown
lmo2196	0.709	*		Multiple Roles	Unknown
lmo1292	0.665			Multiple Roles	Glycerol/lipid metabolism
lmo1995	0.664			Multiple Roles	Unknown
lmo2666	0.564			Multiple Roles	Unknown
lmo1267	0.542			Multiple Roles	Protein folding, transport
lmo0356	0.290			Multiple Roles	Unknown
lmo2695	0.290			Multiple Roles	Unknown
lmo1302	0.290			Multiple Roles	DNA repair, replication, SOS response, transcription regulation, proteolysis
lmo0454	0.290			Multiple Roles	Unknown
lmo1604	-0.254			Multiple Roles	Unknown
lmo0210	-0.291			Multiple Roles	Anaerobic glycolysis
lmo1472	-0.345			Multiple Roles	Protein folding, response to heat
lmo1877	-0.372			Multiple Roles	Unknown
lmo0152	-0.559			Multiple Roles	Unknown
lmo2426	-0.708	*		Multiple Roles	Oxidoreductase
lmo1168	-0.817	*		Multiple Roles	Organic acid metabolism
lmo0191	-0.911	*		Multiple Roles	Unknown
lmo1858	-1.089	*		Multiple Roles	Unknown
lmo1005	-1.407	*		Multiple Roles	Unknown
lmo2437	-1.407	*		Multiple Roles	Unknown
lmo0348	-1.407	*		Multiple Roles	Unknown
lmo2111	-1.938	*		Multiple Roles	Unknown
lmo0024	-1.938	*		Multiple Roles	Integral to membrane, phosphoenol-pyruvate dependant phosphotransferase system

lmo0096	-1.938	*	Multiple Roles	Unknown
lmo1577	-1.938	*	Multiple Roles	Unknown
lmo0898	-1.938	*	Multiple Roles	Unknown
lmo2505	-1.938	*	Multiple Roles	Unknown
lmo0447	-2.325	*	Multiple Roles	Glutamate metabolism
lmo2103	-2.325	*	Multiple Roles	Acetyltransferase
lmo1462	-2.600	*	Multiple Roles	GTP binding
lmo0098	-3.283	*	Multiple Roles	Unknown
lmo0287	-3.283	*	Multiple Roles	Unknown
lmo0866	-3.596	*	Multiple Roles	Unknown
lmo0232	-3.854	*	Multiple Roles	Proteolysis
lmo0640	-4.263	*	Multiple Roles	Unknown
lmo2363	-5.899	*	Multiple Roles	Unknown

5.4 Discussion

While many studies have investigated the protein basis underpinning the acid/alkaline stress, and acid adaptation response in *L. monocytogenes*, very little information is available detailing the alkaline adaptation response (Giotis *et al.*, 2008a; Giotis *et al.*, 2008b). Of the information that is available, all have been derived by methods such as 2-dimensional gel electrophoresis coupled with mass spectrometry (2D-GE-MS). In recent years, rapid advances have been made in the field of proteomics, culminating in the shot-gun approach originally developed by Link *et al.* (1999), known as MuDPIT (detailed in Chapter 4). The work described in this chapter, and that described in Chapter 4, is the first to use MuDPIT in the investigation of the alkaline adaptation response described in *L. monocytogenes.*

Although 2D-GE-MS is an excellent method that has advanced our understanding of functional proteomics, MuDPIT has been demonstrated to have superior detection efficiency due, largely, to the method drawing information directly, in a single process, from a whole cell digest (Koller *et al.*, 2002). However, one of the limitations of MuDPIT (and other proteomics methods) is the difficulty in the analysis of insoluble proteins, many of which constitute a major interest to researchers, particularly the integral membrane proteins. Methods exist that allow the separate solubilisation of this protein fraction for MuDPIT analysis, however these procedures can be lengthy, utilise highly toxic chemicals, and pose the risk of sample loss, biased quantitative results, and contamination (Wolters *et al.*, 2001).

The present work utilised a membrane enriched protein extraction protocol, with the aim of increasing detection of integral and other insoluble cell membrane proteins indirectly by identification of their membrane precursors. Detection of

insoluble proteins was noted, with a number of integral membrane proteins identified. While not comprehensive, the method augmented the protein abundance profile of this fraction obtained from soluble protein extraction alone. Given optimisation of the extraction procedure, increased sample replication, and use in combination with an extraction protocol aimed at proteins present in low abundance, further increases in the resolution of complete shotgun protein profiling should be able to be achieved relatively quickly and simply.

Another potential limitation of MuDPIT is reproducibility of quantitative results. The method has been demonstrated by multiple studies to be highly reproducible (Zybailov, *et al.,* 2005; Washburn *et al.,* 2003; Wu and MacCoss, 2002). However, temporal variation in the preparation and submission of samples, depending on the experiment, can introduce variation in the quantitative data obtained due to a number of factors including the instrument technician, instrument settings, and extraction efficiencies. Such variation can confound quantitative comparisons (Dasch *et al.,* 2009; Matthiesen, 2007).

In the present study, an internal standard, of known concentration, was included in each sample submitted for processing. As part of the data refinement procedure, the spectral counts for each protein identification (including the internal standard, BSA) within each fraction (n = 3) derived from each biological replicate (n = 2), were pooled and normalised against the pooled spectral count of the internal standard for that fraction. This introduced a common protein quantity to the replicates, independent of sample preparation times. As the samples are biological replicates cultured under the same conditions, the peptide spectra should retain common proportions relative to the standard, irrespective of differing spectral values obtained in the raw output. This was observed in the present work with high correlation (R^2=0.9678 and 0.99304) obtained between biological replicates for both pH treatments following normalisation against the internal standard. While large variation between quantitative results of biological replicates should rarely occur (Zybailov, *et al.,* 2005; Washburn *et al.,* 2003), normalisation against an internal standard may validate quantitative results of biological replicates, and contribute to the robustness of quantitative MuDPIT experiments. Based on the results from this study, and the ease of inclusion in experimental preparations, future MuDPIT preparations should routinely include the addition of an internal standard to validate reproducibility, particularly in experiments with a temporal component.

The present work used MuDPIT to investigate the protein abundance profile of alkaline adapted *L. monocytogenes* strain EGD-e. The study confidently identified 534 individual proteins, spanning 96 functional roles, as defined by the JCVI-CMR functional ontology system. This represents a 62% increase on the number of proteins identified by the most comprehensive proteomic study of the alkaline adaptation response in *L. monocytogenes* to date (Giotis *et al.*, 2008).

Results from this study indicate that proteins, peptides and amino acids are fundamental to the alkaline adaptation response of *L. monocytogenes* EGD-e. Increased abundance of proteins associated with valine, leucine and isoleucine metabolism, aromatic amino acid and C5-branched dibasic acid metabolism was observed. This was coupled to a decreased abundance of proteins functioning with lysine biosynthesis, and metabolism of aspartate and glutamate, suggesting that in response to an alkaline environment, the *L. monocytogenes* cells actively acidify the cytoplasm using proteins, while simultaneously decreasing basic elements from this cellular compartment.

The production of aromatic amino acids, which have variable polarity, results in the production of acidic by-products such as 7P-2-dehydro-3-deoxy-D-arabino-heptonate and 3-dehydroquinate. So too, does the degradation of valine, leucine and isoleucine, and C5-branched dibasic acid metabolism, resulting in a range of acidic by-products including 3-hydroxy-3-methyl-2-oxobutanoic acid. As aromatic amino acids have variable polarity, and valine, leucine and isoleucine are non-polar, production, degradation and accumulation of these species may impart a buffering effect while increasing the net acidic state of the cytoplasm. Furthermore, it is known that the polarity of these, and other amino acids (and the side chains of associated proteins), is changed under altered pH conditions (Alberts *et al.*, 2004). As such, increased production of polar amino acid and protein species, or those that undergo polarity shifts conducive to acidifying the cytoplasm, would be advantageous. A similar outcome may also be achieved by decreasing the biosynthesis of lysine, a basic amino acid, and also decreasing the degradation of aspartic and glutamic acids, and decreasing production of amino acids and proteins that do not polarise favourably for buffering the given cytoplasmic pH. Adaptive metabolic patterns such as this have been described, and, in the case of proteins, are believed to represent one of the principal mechanisms for cytoplasmic buffering (Booth, 1985; Padan *et al.*, 2005; Sanders and Slayman, 1982).

Aside from proteins and polypeptides, free amino acids themselves, appear to be directly used as surrogate proton sources as a means of alkaline homeostasis. This has been reported previously (Giotis *et al.*, 2008; Sanders and Slayman, 1982; Booth, 1985) and is supported by evidence obtained in this study. Significantly decreased abundance of proteins associated with peptide catabolism was observed, coupled with significantly increased abundance of peptide transporters and proteolytic elements. It is possible that the identified increase in proteolytic enzymes may fulfil a similar role to the deaminases, widely reported as essential for the alkaline tolerance response in *Escherichia coli* and other bacterial species (Padan *et al.*, 2005; Blankenhorn *et al.*, 1999; Gale and Epps, 1942). These enzymes cleave the amino group from amino acids, thereby directly contributing to cytoplasmic acidification. Deaminases are underrepresented in the *L. monocytogenes* EGD-e proteome, and as such, proteolytic enzymes may be employed to achieve free charged protein fragments. Again, this could contribute to the free amino acid pool, with an associated increase to the net acidification of the cytoplasm.

Direct amino acid, polypeptide and protein based cytoplasmic buffering alone, however, cannot accommodate alkaline homeostasis, as these, along with all cellular mechanisms, form part of a larger biochemical passage within the cell. As such, it should be anticipated that fluxes in products of biochemical mechanisms, including stabilisation processes and direct production of amino acids and proteins, should be observed in alkaline adapted cells. This was observed in the present study.

Protein stabilisation, particularly maintenance of tertiary structure, is concomitant with cellular stability under alkaline conditions. This is evident in the present work by a number of folding and stability proteins identified in increased abundance (two significantly; lmo2069 and lmo1474), including one of viral function (lmo2219), under alkaline conditions. Protein function is a property of the tertiary and quaternary structure. Proteins achieve this structure through positive contributions by weak forces including hydrophobic, electrostatic and Van der Waals interactions; and lose their active conformation when these interactions are lost. These weak forces are easily broken, a trait that affords the plasticity of functional adaptation and regulatory responsiveness characteristic of proteins (Alberts *et al.*,2004). However, alkaline conditions also interfere with these weak interactions, disrupting the tertiary structure of the protein, potentially diminishing or abolishing the biological function of the molecule (Scandurra *et al.*, 2000).

While, altered cytoplasmic pH can be buffered by dissociable H^+ of protein and other macromolecular side chains, given a greater cytoplasmic pH shift, the buffering ability of macromolecules can be overwhelmed leading to denaturation and an abrupt increase or decrease in the cytoplasmic pH. Additionally, some of these proteins are likely to have other roles in alkaline homeostasis. As such, specific proteins that stabilise the tertiary structure of their protein substrates, and, subsequently, their biological activity and polarity, are essential for the cell to maintain both a favourable cytoplasmic pH, and other essential maintenance and repair processes conducive to alkaline homeostasis.

Proteins associated with acetylation of amino acids were highly represented overall, and were increased in abundance, when compared with the control treatment. This included a number of specific amino acid aminoacyl-tRNA synthetases. Acetylases function in the synthesis of acetic acid esters from the parent molecule (substrate). Aminoacyl-tRNA synthetases esterify their corresponding amino acid to their cognate tRNA, forming a charged tRNA, which is then transferred *via* a ribosome onto a growing peptide (Alberts *et al.*, 2004). The increased abundance of aminoacyl-tRNA synthetase can be attributed to increased protein synthesis during alkaline homeostasis, evidenced by the increased abundance and number of proteins associated with translation, and elements regulating the fidelity of translation (e.g. lmo1756). This further supports the importance of proteins and their building blocks in the alkaline tolerance response of *L. monocytogenes.* Notably, residue specificity was observed in terms of increased and decreased amino acid acetylation. Increased abundance of aminoacyl-tRNA synthetase specific for asparagine, proline, serine, cysteine, and ariginine was observed, while decreased abundance of aminoacyl-tRNA synthetase specific for aspartate, phenylalanine, methionine, leucine, lysine, glutamate, isoleucine, valine and alanine was evident. This biased production may represent an attempt to increase protein stability by amino acid substitutions as described by Gromiha *et al.* (1999), as well as the potential for acidic contribution to the cell cytoplasm, indirectly, as part of a more complex pathway, or directly, as part of a polypeptide or protein. Again, this provides evidence for protein production as one of the principal mechanisms for cytoplasmic buffering. In addition, it may be that the creation of a charged tRNA-amino acid, although transient in nature, may be beneficial for the overall net cytoplasmic charge. This would support the notion of a complete cellular response, particularly a metabolic shift, which is directed at pH

homeostasis and avoidance of catastrophic cytoplasmic pH shifts under the prevailing environmental conditions.

The results presented provide evidence for an alkaline adaptation-induced shift in energy metabolism. The production of adenosine triphosphate (ATP), protons and acidic by-products from glycolysis and the pentose phosphate shunt appear to have a role in alkaline homeostasis. Increased protein abundances associated with both of these cycles were observed. This included proteins involved with the phosphotransferase system (PTS), associated with transporting glycolysis substrates into the cell (e.g. lmo2475, lmo0427). This was coupled to a decrease in proteins associated with the production of tricarboxylic acid cycle substrates (e.g. lmo2052, 1566). These apparent alterations to flow of substrates through linked pathways could serve a number of functions under alkaline conditions. The pentose phosphate shunt could serve to increase production of reducing equivalents (NADH) directed at fatty acid biosynthesis, the electron transport chain (ETC) and a wide range of other cellular processes (including nucleotide biosynthesis – a five carbon sugar), needed for cell proliferation (DNA) and protein expression *via* messenger RNA.

Increased proteins associated with fatty acid biosynthesis were observed in the present study; however, it was coupled to a decrease in other proteins of the same biological function. While fatty acids are known to have a role in the pH tolerance response of *L. monocytogenes*, it is reported that the type, rather than number, of fatty acid is what imparts the protective effect (Giotis *et al.*, 2007b). Protection is thought to result from membrane/cell wall alteration, manifested through alteration of fatty acid chain length and branching, aimed at increasing cell envelope integrity (Giotis *et al.*, 2007b). As such, the differences in protein abundances associated with fatty acid biosynthesis may reflect the type of fatty acids being produced, which, in turn, may reflect the cellular response induced by the pH of the culture environment.

The pentose phosphate shunt also produces precursors that go on to produce aromatic amino acids and nucleic acids and nucleotides. These latter products appear to be important in the alkaline adaptive response in *L. monocytogenes* EGD-e, given the increased abundance of proteins (two significantly; lmo0219 and 1856) associated with nucleotide salvage (lmo0219, 1856, 1954 and 1463) and biosynthesis (lmo1874, 1771, 1835, 0055 and 2758). These elements are associated with cell wall biosynthesis, molecular signalling, regulation of cellular processes and as intermediates in energy production. These molecules may be increased in response to increased cell wall biosynthesis. Interestingly, at elevated pH, free nucleic acids are

predominantly ionised. As such, increased production may be directed at increasing the cell wall acidity, aimed at countering the external pH and minimising damage to the outer cell wall. Furthermore, this may also directly contribute to cytoplasmic acidity (Cho and Evans, 1991).

The importation of sugars via the PTS system could serve to buffer the cytoplasm, while increasing substrates for glycolysis, and, therefore, the associated acidic by-products. Furthermore, the PTS system, along with other transport and biochemical systems, could facilitate the removal of free phosphate ions from the cytoplasm, produced through a number of biochemical anabolic and catabolic pathways (particularly the consumption of ATP), thereby contributing to increased cytoplasmic acidification through glycolysis and the removal of an increasing pool of cytoplasmic anionic by-products.

A number of biochemical pathways produce by-products that are associated with the electron transport chain. This multi-step energy generating system involves a number of protein components that transfer electrons from the initial NADH and succinate donors (generated by the tricarboxylic acid (TCA)/pentose phosphate/glycolysis pathways, and the limited fatty acid degradation observed in the current study), culminating in energy production by an ATP synthetase (proton pump) powered by a proton motive force (Alberts *et al.*, 2004). However, in the present study, diminished TCA activities and decreased ubiquinone biosynthetic enzymes were detected (lmo1673), along with decreased abundance of proteins associated with the ATP-proton motive force (lmo0092, 0088, 2530, 2532 and 2528).

A diminished ATP-proton motive force would appear to oppose, to some extent, any cytoplasmic acidification process, as the proton pump (driven by the proton motive force) expels protons into the cytoplasm in the generation of energy (ATP) via an ATP synthetase according to the formula:

Adenosine diphosphate + Phosphorous + 4 protons ↔ Adenosine triphosphate + water + 4 protons

With this in mind, and considering the decreased proton motive force and abundance of ubquinone, it is possible that under alkaline conditions, at least two key energy shift processes are occurring: 1) one of the three levels of electron donation into the ETC is inhibited, contributing to cytoplasmic acidification by increasing the succinate and NAD^+ pool within the cytoplasm and, 2) another force is causing loss of protons from the periplasmic space, decreasing the flow back through the ATP

synthase, and resulting in a net loss of protons from the cytoplasm. This would lead to a decrease in the proton motive force, with a reversion to passive proton transport (reversal of the previously mentioned formula) in order to maximise proton retention within the cytoplasm. A decrease in ATP synthase activity in cells grown at alkaline pH has been demonstrated previously (Booth, 1985).

The mechanism driving this net loss may be explained by the high cell surface pH (low proton concentration) derived from the alkaline culture media. This concept is described in a microbial attachment model introduced by Hong and Brown (2010). In their model, they suggest that a charge-regulation effect may be induced by the environmental pH (attachment surface in the original study) in proximity to the cell surface, resulting in a net migration of protons from the cell periplasmic space to the cell surface and out of the cell. This migration of protons would shift the equilibrium of the ATP synthase phosphorylation reaction, and given prolonged exposure to the alkaline conditions, proteins associated with the proton motive force are decreased (Booth, 1985; Hong and Brown, 2010; Figure 5.4). The associated deficit in energy (ATP) production may be offset by the observed increase in glycolysis and the pentose phosphate pathways (i.e. substrate level phosphorylation).

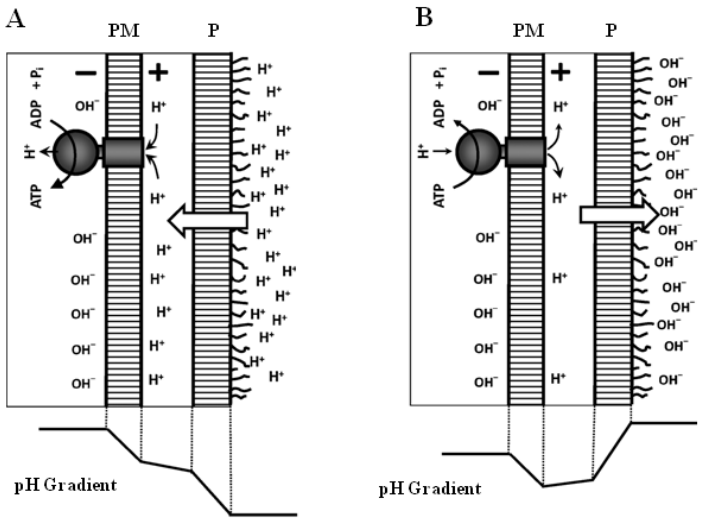


Figure 5.4 The proposed pH gradient model introduced by Hong and Brown (2010), adapted to explain the decreased proton motive force observed in alkaline adapted *L. monocytogenes* EGDe cells in the current study. The figure represents a cross-section detailing the plasma membrane (PM) and peptidoglycan (P) and ATP synthase of a Gram positive cell exposed to acidic (A) and alkaline (B) environmental conditions. A charge-regulation effect may be induced by the environmental pH (culture media in the current study) in proximity to the cell surface, resulting in a net H^+ migration into (A) or out of (B) the cell periplasmic space in a passive equilibration process. The migration of H^+ subsequently effects the equilibrium of the ATP synthase phosphorylation reaction, altering the direction in which the protons flow, and subsequently, the generation or consumption of ATP. Given prolonged periods under these conditions, proteins associated with generation of a proton motive force may be down-regulated.

The combination of these mechanisms of acidification, including the charge regulation effect, could ultimately lead to an enhanced reducing environment within the cytoplasm, with a subsequent increase in reactive oxygen species through electron leakage (Schafer and Buettner, 2001). This was suggested in the current study by significantly increased abundance of lmo1407. This protein, generally associated with anaerobic respiration, has been observed to increase under oxidative stress in the presence of increased reactive oxygen species (Feng *et al.,* 2009). It is thought that an oxygen – limiting state may be induced under these environments, inducing up-regulation of lmo1407 (Feng *et al.,* 2009).

The imbalance in the redox state of the cytoplasm could be a necessary adaptive sacrifice, given the net decrease in abundance of proteins associated with redox homeostasis. Given this, and the proposed charge-regulation effect, an energy generation shift during alkaline homeostasis towards glycolysis and other pathways that directly or indirectly contribute to cytoplasmic acidification via fermentation, may represent a compromise, not an ideal metabolic situation. Such inefficient use of energy substrates might be expected to result in reduced biomass/cell yield (i.e. stationary phase observed at a lower cell density). This was observed in turbidimetric measurements obtained from alkaline adapted relative to steady state (pH7.3) acclimated cells in the present study. It may be that the cells, although alkaline adapted, are significantly stressed. As such, it could be expected that cell wall modifications and repairs, and induction of transporters, would increase to compensate for the redox imbalance generated, and the proposed charge-regulation effect. This notion was supported by the protein expression observed in the alkaline adapted *L. monocytogenes* cells.

The abundance of transport and binding proteins was both increased, and decreased, in alkaline adapted *L. monocytogenes* EGD-e. However, although regarded as the dominant mechanism underpinning the control of cytoplasmic pH, no specific cation/H^+ antiporters were detected in the present study. These transporters exchange internal cations for external protons, thereby acidifying the cytoplasm (Hunte *et al.,* 2005). It is possible that the extraction method used in this study failed to isolate these elements due to their high insolubility; however a number of other transport proteins showed increased abundance, including cation transmembrane transporters (cobalt, mercury and copper).

An alternative reason for their absence could be that they constitute part of the alkaline stress and early adaptive response only, representing a transient mechanism

directed at "emergency" acidification of the cytoplasm. This could be a deliberate cessation of activity, resulting from the physical, energetic or external influences such as the proposed charge-regulation effect. The energy expended to maintain a more acidic cytoplasmic pH than the extracellular environment through cation cycling may be too high. As such, the Na^+/H^+ system (and others) may be down regulated, in favour of other, less costly, coupled systems. This has been indirectly observed in past studies of other microorganisms (e.g. Booth, 1985).

The cells in the present study were adapted over a period of seven days. As cytoplasmic pH reaches balance the cellular mechanisms may shift towards other, less demanding forms of cytoplasmic acidification, that are *coupled* to other essential processes. Significantly increased amounts of lmo0507 were identified, along with lmo1389, 0181 and 2193. Lmo0507 is an integral membrane sugar-hydrogen symporter. Four other integral membrane sugar-hydrogen symporters were identified in increased abundance, highlighting an important role in alkaline homeostasis. It is possible that the increased presence of these symporters may serve multiple complementary functions, forming part of an alternative, energy conserving cytoplasmic acidification process. One of these is the direct import of hydrogen cations, directly contributing to the net acidification of the cytoplasm. Secondly, the transporters import carbohydrates that may feed into the glycolysis and pentose phosphate pathways, creating energy with the associated production of acidic and cellular maintenance by-products.

Aside from cytoplasmic acidification, processes associated with maintenance of cellular structural integrity, and other adjustments conducive to adaptation to an alkaline environment would be expected. Proteins associated with adaptation to stress, pathogenesis, and maintenance of cell wall integrity were observed in the current study. Specific stress adaptation proteins were significantly increased in abundance, including lmo2217, 1601 and 1879, predominantly functioning as molecular chaperones and stabilisation elements. Interestingly, genomic DNA appeared relatively unaffected by alkaline adaptation, with regulation of DNA replication significantly increased, while many DNA repair proteins decreased in abundance. This likely reflects the physical properties of DNA, with increased stability of DNA known to occur under alkaline conditions (Alberts *et al.,* 2004).

Notably, the abundance of cell surface proteins was reduced in alkaline adapted cells. This included lmo0690 (flagellin), which is known to be repressed at 37°C by MogR (Peel *et al.,* 1988; Shen and Higgins, 2006). However, a range of

other virulence proteins, including lmo0433, lmo0394 and lmo0202 were also decreased. This observation has been noted recently, in a comprehensive study comparing transcription in *L. monocytogenes* between a saprophytic existence and the transition to virulence (Toledo-Arana *et al.,* 2009). Those authors noted that *L. monocytogenes* modified its surface and surface appendages in response to an intestinal environment, which had an elevated pH of approximately 8.0 (Toledo-Arana *et al.,* 2009; Sleator *et al.,* 2009). A notable exception to the reduced abundance of cell surface proteins observed in the current study was a putative cell surface adhesion protein (lmo0880). Increased expression of this protein under stress conditions has been described before, however its specific function remains unknown (Kazmierczak *et al.,* 2003).

Particularly interesting was the observation that many of the cell envelope precursors to the surface associated motility and pathogenesis elements were present in increased amounts, including lmo0333, lmo0204, and lmo0713. It is possible that the expression of flagellar basal body (lmo0713) may represent early induction of a MogR *de-repression* process induced by "gastrointestinal tract-like" conditions (elevated pH) similar to that suggested by Sleator *et al.* (2009). If so, the increased abundance of surface virulence protein precursors may represent an early transitional stage to virulence, constitutively induced with *reinduction* of motility. Alternatively, the increased abundance of cell wall surface protein precursors may simply reflect a compromise associated with regulation of cell shape and cell division processes, as evidenced by the increased abundance of proteins associated with cell wall and shape regulatory and biogenic functions. This has (indirectly) been described before in *L. monocytogenes*, with altered (elongated) phenotypes observed by electron microscopy after exposure to alkaline culture conditions (Giotis *et al.,* 2007). As such, the expression of the cell surface components of these virulence determinants may be inhibited or repressed as the cell attempts to adjust, maintain (or loses control of) the correct morphology and effect successful cell division. Again, alteration to the cell wall may reflect an attempt to counteract the proposed charge - regulation effect, described in Figure 5.4. This is supported in the present study by increased biosynthesis of peptidoglycan, cell wall stabilisers (lmo0197) and diaminopimelate (lmo1435), and likely represents increased efforts to maintain cell wall integrity and structural adjustment (Wehrmann *et al.,* 1998).

The altered expression of proteins associated with cell division observed in the present study is of particular interest. Increased abundance of DivIVA (lmo2020;

significant increase), lmo2033 (FtsA; significant increase) and lmo0220 (FtsH; significant increase) was coupled to a decrease in lmo2032 (FtsZ) and lmo2790 (ParB). In *Bacillus subtilis*, FtsZ initiates cell division, while ParB is involved in chromosome partitioning, FtsA accumulates near the septum during cell division, and DivIVA is a cell division protein associated with regulating septum localisation during division. It is thought that these proteins have a similar role in *L. monocytogenes*. Increased abundances of FtsA and DivIVA within the cell have been associated with an elongated phenotype in *B. subtilis* (Wehrl *et al.*, 2000; Cha and Stewart, 1997). As such, and given the functional similarities the molecules have in *L. monocytogenes*, it is likely that these elements result in the elongated phenotype that has been described for *L. monocytogenes* cells subjected to alkaline culture conditions (Giotis *et al.*, 2007a). Furthermore, this altered phenotype has been shown to decline after extended periods of time subjected to alkaline conditions and is reversible if the alkaline conditions are removed (Giotis *et al.*, 2007a). It is possible that the altered cell phenotype observed under alkaline conditions results from differential expression of the cell division elements FtsA, FtsH, FtsZ, DivIVA and ParB (and others). The reasons underpinning this response are unclear. However they may also be the result of adaptive metabolic shifts, including energetic adjustments directed towards cytoplasmic acidification, and cell wall adjustment aimed at counteracting the charge – regulation effect of the extracellular environment. It is possible, that, after an extended period of time subjected to alkaline pH, the *L. monocytogenes* cell wall is altered enough (through addition of peptidoglycan and other factors) to cope with the prevailing conditions. As such, surface virulence proteins are up – regulated again. This may represent a normal part of the pathogenesis process, applied to counter the alkaline villi of the gastrointestinal tract and ready the cell for intracellular life and the phagocytic respiratory burst.

To conclude, membrane protein enriched MuDPIT analysis has provided insight into alkaline adaptation by *L. monocytogenes* EGD-e, augmenting current knowledge of the proteome changes accompanying this response. Results from this study suggest that alkaline pH homeostasis in *L. monocytogenes* EGD-e results from multiple regulatory mechanisms, allowing for finer control over cellular pH. These mechanisms appear to be interconnected, driven by the necessity to increase cytoplasmic acidity, maintain cellular integrity, and minimise other physical effects imparted by the extracellular environment.

A key component of this response appears to be direct cytoplasmic acidification through the production, retention and importation of polar or charged proteins, peptides and amino acids. Furthermore, systems are mobilised that facilitate stabilisation of these and other regulatory proteins presumably to prevent pH induced conformational changes that may lead to loss of function. This is coupled with an adaptive shift in energy metabolism that increases production of acidic by-products, ATP and reducing equivalents, to compensate for inhibition of other energy production pathways that are physically influenced by the extracellular pH environment. This includes restriction of the electron transport chain and inversion of the proton motive force to conserve protons that are being lost due to the proposed charge-regulation effect, and increase the pool of oxidised reducing equivalents.

It is hypothesised that to conserve energy, more energy efficient symport systems (such as the sugar – hydrogen) are increased to substitute the energy expensive, stress associated cation/proton transporters, providing protons to acidify the cytoplasm while also importing sugars that may be utilised in the adapted energy metabolisms. Cell wall modifications, including decreased cell surface structures and increased biosynthesis of peptidoglycan, diaminopimelate, and different fatty acid isoforms, are implemented to maintain cellular integrity and attempt to offset the charge-regulation effect. This could lead to an altered, elongated phenotype, induced by differential production of key cell division proteins, resulting from alternative resource allocation caused by and directed at minimising the charge regulation effect. An elongated phenotype under alkaline culture conditions has been described previously (Giotis *et al.*, 2007a).

L. monocytogenes undergoes profound physiological shifts during the alkaline adaptation response. It is likely that a similar response may be induced during passage through the mammalian small intestine which is maintained at approximately pH8.0. As such, these changes must, to some extent, represent a normal physiological shift. Given *L. monocytogenes*' ability to revert to a normal physiological and phenotypic state (e.g. from an elongated phenotype after exposure to alkaline conditions) these adaptive mechanisms would seem to correspond to a transient survival tactic. Passage through the mammalian gastrointestinal tract subjects *L. monocytogenes* to a broad spectrum of pH over a long period (≈6 hours). Adjusting the cell physiology during this transit may allow for survival, persistence, attachment, and, with concomitant expression of surface virulence proteins, allow infection and "ready" the cells for intracellular life and the phagocytic respiratory

burst. As the reservoir of *L. monocytogenes* includes soil, forage and water, surviving the passage through the mammalian GIT provides a means of dissemination and dispersal. As such, physiological adaptations conducive to surviving this passage may afford the organism an ecological advantage.

CHAPTER 6
GENERAL DISCUSSION

Persistent contamination of food processing environments by *Listeria monocytogenes* represents a threat to public health and can have serious economic implications for the contaminated facility. Persistent *L. monocytogenes* strains within food production and processing facilities increase the likelihood of food product contamination. The controls that are in place to prevent microbial contamination of both the environment and food products within these facilities physiologically stress *L. monocytogenes*, and stress resistance is believed to contribute or select for environmental persistence. This is an important concept as a number of studies have shown that exposure to, and the ability to resist, physiological stress can augment the virulence of *L. monocytogenes* (Anderson *et al.*, 2007; Gahan *et al.*, 1996; Gandhi and Chikindas, 2007). Consequently, persistent *L. monocytogenes* strains in factories may constitute a physiologically toughened population with increased propensity to cause human disease.

The primary objective of this study was to define biological mechanisms that facilitate persistent contamination of food production and processing environments by *L. monocytogenes* strains. This was approached by characterisation of persistent food factory contaminants using multilocus sequence typing (MLST), followed by investigation in representative strains of stress resistance mechanisms known to facilitate or aid in persistent contamination. These mechanisms included the ability to produce biofilm and to adapt to alkaline pH environments. The results of this study suggest that multiple persistent *L. monocytogenes* phenotypes exist, that have an increased ability to produce biofilm and with increased stress resistance, induced by the environmental conditions to which the cells are subjected. Strains recovered as persistent contaminants showed similar biofilm production and stress resistance responses to a sporadic factory contaminant; however the persistent strains appear to have a greater propensity to undergo a shift conducive to alkaline adaptation.

In this study persistent food factory contamination by *L. monocytogenes* was confirmed and the strains defined using MLST. The persistent contaminants corresponded to two previously described MLST sequence types (ST), ST – 3 and ST – 155; associated with 32 and 13 individual cases of listeriosis in Australia respectively between 2001 and 2004. In addition the typing indicated that the factory suffered systemic contamination by *L. monocytogenes*, with the same ST's

distributed throughout multiple areas of the facility. This is indicative of persistent contamination, implicates an internal contamination source and supports the assignment of persistence status to those *L. monocytogenes* strains. Characterisation of these strains provided an opportunity for further investigation of persistent and sporadic *L. monocytogenes* strains. The strains studied have been implicated in human infections and were recovered from a food environment, and all are likely to have been subjected to similar environmental conditions, including stress.

So to what extent are the persistent strains distinct from the environmentally sporadic strains? Comparison of biofilm production by 95 *L. monocytogenes* isolates obtained from a range of sources, including the factory contaminants, highlighted a temperature specific biofilm response. This involved increased biofilm production by clinical *L. monocytogenes* isolates at elevated temperatures and increased biofilm production by serotype 1/2a strains irrespective of temperature. Further to this, all of the factory *L. monocytogenes* strains, including persistent and sporadic contaminants, produced a similar level of biofilm, particularly when subjected to pH and low temperature challenge. This lead to the hypothesis that an association exists between temperature, physiological stress, virulence and biofilm production mechanisms in *L. monocytogenes*, with overlap between the survival (biofilm production) and virulence responses, dictated by the presence of conditions similar to those found within a mammalian host, stress and other environmental influences. It is possible that biofilm production and virulence may be induced by similar environmental cues, and that given the appropriate stimuli, *L. monocytogenes* may initiate either a biofilm or virulence response. In particular, the homogeneous biofilm production response of the persistent and sporadic factory contaminants implies that the level of biofilm production by *L. monocytogenes* may be a characteristic of the environment in which they have adapted, and supports the notion that biofilm production may be one of a number of characteristics facilitating persistent environmental contamination.

Evaluation of the protein expression profiles of persistent and sporadic alkaline-adapted *L. monocytogenes* factory contaminants provided evidence for the mechanisms underpinning this response. Active acidification of the cytoplasm, stabilisation of regulatory processes, cytoplasmic buffering, enhanced cell wall maintenance and a shift in energy metabolism were inferred. Furthermore, there appeared to be overlap between proteins associated with the virulence, alkaline and growth phase induced stress response.

Importantly, the shift in energy metabolism inferred from the protein profile of alkaline-adapted *L. monocytogenes* showed similarities to the charge – regulation model proposed by Hong and Brown (2010) (*see* Figure 5.4). When coupled to the observed increase in virulence determinants observed in the protein expression profiles, this supports the hypothesis that overlap exists between the stress response, biofilm production and virulence in *L. monocytogenes* (*see* Figure 3.8). Proteome comparison of alkaline-adapted persistent and sporadic *L. monocytogenes* factory contaminants suggests that the persistent strains may be better able to initiate the physiological adjustments conducive to alkaline adaptation. When considered with the biofilm production results it seems that two persistent phenotypes may exist; one with an increased propensity to produce biofilm, and another with an increased propensity for stress resistance, with the latter likely to be associated with increased virulence.

It is possible that microenvironments within food production / processing plants can contribute to the development of physiologically robust *L. monocytogenes* strains. Results from this study suggest that the physiological robustness can manifest as a "concealment" phenotype, characterised by the production of biofilm with subsequent protection from environmental challenge; and a resistant phenotype, characterised by an innate physiological resistance to environmental challenge, with overlap between the two. These may be inadvertently selected by the complexity of food production/processing facilities leading to concentration fluxes of cleansing/sanitising agents, and other growth limiting challenges. If exposed to non-lethal stress conditions, the resistant phenotypes may be induced, affording cross protection against other stresses, and could facilitate environmental persistence. The combination of variable stress resistance and biofilm production, coupled with other strain specific physiological attributes, may confound the characterisation of a single specific, environmentally-persistent phenotype. It seems likely that multiple persistent phenotypes exist and can be developed, depending on the attributes of each *L. monocytogenes* strain and the microenvironments and associated conditions to which they are exposed.

The control of persistent food processing factory contamination by *L. monocytogenes* remains a serious public health risk. The incidence of listeriosis is increasing in Australia (CDA, 2010; *see* Figure 1.1), despite advances in our knowledge of the biology and ecology of *L. monocytogenes*. This is likely to continue with the recent increased public interest (and subsequent economic

attractiveness) of small scale “cottage” food production industries, including ready – to – eat products including processed meats (the food type accounting for the greatest number of Australian outbreaks; CDA, 2010) that are manufactured under poorly controlled conditions. Further to this, there is an increasing proportion of the population with predisposition to listeriosis through reduced immunity. Although “low levels equals low risk” (Chen *et al.*, 2003), it must be remembered that a single case of listeriosis is unacceptable, irrespective of any predisposing condition. Targeted and improved sanitation is required to prevent contamination and eliminate persistent *L. monocytogenes* strains from the food industry. Increased knowledge of the biology of *L. monocytogenes*, particularly those attributes that allow the organism to contaminate and persist in food factories, may allow targeted control, and guide preventative strategies directed at the organism. This is essential for effective protection of the food supply chain from *L. monocytogenes*.

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APPENDIX 1
GENERAL TECHNICAL REAGENTS AND METHODS

A1.1 Extended Storage and Recovery of Bacterial Isolates

Extended storage and recovery of bacterial isolates was achieved using the Protect preservation system (Oxoid, Adelaide, Australia) according to manufacturer instructions. The storage vials were labelled and kept at − 80°C. Recovery of bacterial isolates from this system was performed according to manufacturer instructions using brain – heart infusion agar supplemented with 0.1% sodium pyruvate (Appendix 2.3), followed by incubation at 30°C for 24 hours. All isolates were confirmed as *Listeria monocytogenes* by subculture onto PALCAM agar (Appendix 2.4).

A1.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise all PCR products. The volume and percentage gel required depended on the size of the PCR product produced (Table A1.1).

Table A1.1 Percentage of agarose gel required for efficient separation of linear DNA molecules (Sambrook *et al.* 1989).

% Agarose (w / v)	Linear DNA Molecule Size Range (kb)
0.3	5 - 60.0
0.6	1 - 20.0
0.7	0.8 - 10
0.9	0.5 - 7.0
1.2	0.4 - 6.0
1.5	0.2 - 3.0
2.0	0.1 - 2.0

The required amount of agarose (Sigma Genosys Pty. Ltd, Sydney, Australia) was added to 150 mL of 1 × trisethylenediamine tetra-acetic acid buffer (TE; Appendix 1.3) in a conical flask. The mixture was heated in a microwave oven, stirring regularly, to dissolve the agarose. When the agarose was dissolved, the solution was allowed to cool to approximately 50 - 60°C.

Ethidium bromide (Sigma Genosys Pty. Ltd, Sydney, NSW, Australia) was added (7.5 μL of a 10 mg / mL stock solution for a 150 mL gel). The solution was poured into a gel cast and placed in an electrophoresis tank (Scie – Plas, Crown Scientific, Kewdale Western Australia). An appropriately slotted gel comb was fitted and the gel was allowed to set completely at room temperature. Once the gel was set, the gel comb was gently removed and 1 × TE was added to the electrophoresis tank until it covered the gel. Molecular weight marker was prepared by combining 2 μL of GeneRulerTM 100 bp DNA ladder (Fermentas, Brisbane Australia) with 2 μL 6 × loading dye solution and 8 μL of deionised water (18.2 mΩ). Each PCR product to be visualised was prepared for loading by combining 2 μL of 6 × loading dye solution with 10 μL of PCR product. Molecular weight marker and the prepared PCR product was loaded into individual gel lanes, the electrophoresis tank was connected to an electrical supply, and the gel was run at 2 volts cm-1. Following electrophoresis, the gel was removed and viewed under ultra violet light using an EDAS 290 ultraviolet transilluminator (Kodak, Sydney, Australia).

A1.3 Trisethylenediamine Tetra-Acetic Acid Buffer (T.E.)

Hydroxymethylamine (100 mM) (Sigma, Sydney Australia)	50 mL
Ethylenediamine tetra-acetic acid (E.D.T.A.) (10 mM) (Sigma, Sydney Australia)	50 mL
Ethylenediamine tetra-acetic acid (E.D.T.A.) (0.1 mM)	900 mL

Combine the100 mM hydroxymethylamine and the 10mM E.D.T.A. and adjust the pH to 8.0 using hydrochloric acid. This reduces depurination of the stored DNA. Make a 1:10dilution of the prepared T.E. buffer with the 0.1mM E.D.T.A. This reduces the interaction of the E.D.T.A with downstream applications of the stored product.

A1.4 Bovine Serum Albumin Standard – 500 μg/mL Stock Solution

Albumin, bovine serum, fraction V (Sigma - Aldrich, Castle Hill, Australia)	0.05 g
Deionised water (18.2 mΩ)	100 mL

Autoclave water at 121°C for 20 minutes. Aseptically add albumin powder and stir to dissolve. Store out of sunlight at 2 - 4°C.

A1.5 Osmium Tetroxide (1%)

4 % Osmium tetroxide solution (ProSciTech, Queensland, Australia)	5 mL
Single distilled water	15 mL

Osmium tetroxide is highly toxic. Perform all steps in a fume hood and wear protective clothing. Combine all of the ingredients and gently mix.

A1.6 Phosphate Buffered Saline (10× stock)

Sodium chloride (NaCl) (Sigma - Aldrich, Castle Hill, Australia)	80 g
Potassium chloride (KCl) (Sigma - Aldrich, Castle Hill, Australia)	2 g
Sodium phosphate (KH_2PO_4) (Sigma - Aldrich, Castle Hill, Australia)	2.4 g
Single distilled water	1000 mL

Combine ingredients and dissolve by stirring using a magnetic stirrer. Autoclave at 121°C for 20 minutes. The 10 × stock has a pH of ≈6.8. When diluted to 1 × the pH adjusts to ≈ 7.4. Fine adjustments can be made using hydrochloric acid or sodium hydroxide.

A1.7 Cacodylate Buffer Stock Solution (0.2 M)

Sodium cacodylate trihydrate (ProSciTech, Queensland, Australia)	20.15 g
Hydrochloric acid (glacial) (Sigma - Aldrich, Castle Hill, Australia)	0.1 mL
Single distilled water	250 mL

Cacodylate is a known carcinogen. Perform all steps in a fume hood and wear protective clothing. Combine all of the ingredients and gently mix. Adjust pH to 7.4 if necessary using hydrochloric acid or sodium hydroxide.

A1.8 Cacodylate Buffer with 6% Sucrose and 0.1% Calcium Chloride (0.1 M)

0.2 M cacodylate stock solution (A3.1)	250 mL
RNAase free sucrose (Sigma - Aldrich, Castle Hill, Australia)	30 g
Calcium chloride (Sigma - Aldrich, Castle Hill, Australia)	0.05 g
Single distilled water	250 mL

Cacodylate is a known carcinogen. Perform all steps in a fume hood and wear protective clothing. Combine all of the ingredients and gently mix.

A1.9 8 M urea, 100 mM Ammonium Bicarbonate

Urea (Sigma - Aldrich, Castle Hill, Australia)	2.7 g
400 mM Ammonium bicarbonate stock solution (Appendix 1.13)	1.25 mL
Deionised water (18.2 mΩ)	≈4 mL

Dissolve urea in the ammonium bicarbonate stock. Adjust the volume 5 mL with deionised water. Autoclave at 121°C for 20 minutes.

A1.10 50 mM Ammonium Bicarbonate, 1 mM Calcium Chloride in Water

400 mM ammonium bicarbonate solution (Appendix 1.13)	500 µL
Deionised water (18.2 mΩ)	3.45 mL
100 mM calcium chloride solution (Appendix 1.14)	40 µL

Mix the ammonium bicarbonate solution with the water. Add the calcium chloride solution. Autoclave at 121°C for 20 minutes.

A1.11 High Pressure Liquid Chromatography buffers

These buffers were prepared at the Central Science Laboratory, UTAS.

A	0.1 % Formic Acid, 0.1% Triflouro Acetic Acid
B	5% Acetonitrile + 0.1 % Formic Acid
C	90% Acetonitrole + 0.1 % Formic Acid
D	500 mM Ammoium Acetate in 5% Acetonitrile

A1.12 The five step MuDPIT gradient program used for this study. A, B, C and D represent the buffers outlined in A1.11.

Step	Time (min)	%A	%B	%C	%D
Loading	0	100			
	10	100			
1	0		100		
	5		100		
	10		85	15	
	70		55	45	
	80			100	
	90			100	
	90.1		100		
	95		100		
2	0		90		10
	5		90		10
	5.1		100		
	20		85	15	
	75		75	25	
	125		50	50	
	125.1		100		
	130		100		
3	0		80		20
	5		80		20
	5.1		100		
	20		85	15	
	75		75	25	
	125		50	50	
	125.1		100		
	130		100		

4	0	60		40
	5	60		40
	5.1	100		
	20	85	15	
	75	75	25	
	125	50	50	
	125.1	100		
	130	100		
5	0			100
	15			100
	15.1	100		
	25	80	20	
	90	55	45	
	95		100	
	115		100	
	115.1	100		
	130	100		

A1.13 400 mM Ammonium Bicarbonate Stock

Ammonium Bicarbonate (Sigma - Aldrich, Castle Hill, Australia)	1 g
Deionised water (18.2 mΩ)	25 mL

Dissolve ammonium bicarbonate in the deionised water. Autoclave at 121°C for 20 minutes.

A1.14 100 mM Calcium Chloride Solution

Anhydrous calcium chloride (Sigma - Aldrich, Castle Hill, Australia)	0.27 g
Deionised water (18.2 mΩ)	25 mL

Dissolve calcium chloride in the deionised water. Autoclave at 121°C for 20 minutes.

APPENDIX 2
BACTERIOLOGICAL MEDIA

A2.1 Brain – Heart Infusion Agar

Brain – heart infusion broth (Appendix 2.2)	1000 mL
Technical agar number 3 (Oxoid, Adelaide, Australia)	15 g

Dissolve agar in brain – heart infusion broth. Autoclave at 121°C for 20 minutes.

A2.2 Brain – Heart Infusion Broth

Brain – heart infusion media (CM225; Oxoid, Adelaide, Australia)	37 g
Single distilled water	1000 mL

Dissolve brain – heart infusion media in 50 mL of the single distilled water. Add remaining single distilled water and stir using a magnetic stirrer until homogenised. Autoclave at 121°C for 20 minutes.

A2.3 Brain – Heart Infusion Agar Supplemented with 0.1% Sodium Pyruvate

Brain – heart infusion broth (Appendix 2.2)	1000 mL
Technical agar number 3 (Oxoid, Adelaide, Australia)	15 g
Sodium pyruvate (Sigma – Aldrich, Castle Hill, Australia)	1 g

Dissolve agar in brain – heart infusion broth. Add sodium pyruvate and stir using a magnetic stirrer until homogenised. Autoclave at 121°C for 20 minutes.

A2.4 PALCAM Agar

PALCAM agar base (CM0877; Oxoid, Adelaide, Australia)	34.5 g
Single distilled water	500 mL
PALCAM selective supplement (SR0150E; Oxoid, Adelaide, Australia)	1 vial

Dissolve palcam AGAR BASE in 50 mL of the single distilled water. Add remaining single distilled water and stir using a magnetic stirrer until homogenised. Autoclave at 121°C for 20 minutes. Cool to 50°C and add PALCAM selective supplement. Mix well.

APPENDIX 3

CLEANED ALLELE SEQUENCES

abcZ -5: (Strains DS_14, 31, 53, 63, 88 and PRD5)

AAATCAACGAACAGAATGCGTATAGGGCTTTTCCGCAAGATGGAAAAACTATCGATTCG
TTTCTTCGATAGCCGCAATGATGGCGAAATGCTTAGCCGCTTCACTAGCGACTTAGATAA
TATTTCCAATACACTAAACCAAGCATTGATCCAAGTACTATCCAACGTCGCGCTAATGAT
TGGTGTTATCATCATGATGTTCCAACAAAACGTGGAACTAGCCTTCGTTACTCTAATATCT
GCTCCATTTGCAATTATTATTGCGACAGTGATTATTCGAAAAGCACGTAAATTCGTTGAT
GTTCAACAAGATGAACTAGGCGTACTTAACGGCTACATTGACGAAAAAATCTCTGGACA
AAAAATCATTATCACAAATGGTTTAGAAGAAGAAACAATTGACGGCTTTGTTAAACAAA
ACAATATCGTTAAAAACGCCACTTATAAAGGGCAAGTTTACTCCGGTTTACTTTTCCCAA
TGATGCAAGGTATTTCCCTATTAAATACAGCTATCGTTATCTTCTTCGGTGGATGGTTA

abcZ -4: (Strains DS_25, 81, 82, 84 and 85)

AAATCGACGAACAGAATGCGTATAGGGCTTTTCCGCAAGATGGAAAAACTATCAATCCG
TTTCTTCGATAGCCGCAATGATGGCGAAATGCTTAGCCGCTTCACTAGTGACTTGGATAA
TATTTCCAACACACTAAACCAAGCATTGATCCAAGTACTATCCAACGTCGCGCTAATGAT
TGGTGTTATCATCATGATGTTCCAACAAAACGTGGAACTAGCCTTCGTTACTCTAATATCT
GCTCCATTTGCGATTATTATTGCGACAGTGATTATTCGAAAAGCCCGCAAATTCGTTGAT
ATTCAACAAGATGAACTAGGCGTACTTAACGGCTACATTGACGAAAAAATCTCTGGTCA
AAAAATTATTATCACAAATGGCTTAGAAGAAGAAACAATTGACGGCTTTGTTAAACAAA
ACAATATCGTTAAAAACGCCACTTACAAAGGTCAAGTTTACTCCGGTTTACTTTTCCCAA
TGATGCAAGGTATTTCCCTATTAAATACAGCTATCGTTATCTTCTTCGGTGGATGGCTA

abcZ -7: (Strains DS_68 and 80)

AAATCGACGAACAGAATGCGTATAGGGCTTTTCCGCAAGATGGAAAAACTATCGATTCG
TTTCTTCGATAGCCGCAATGATGGCGAAATGCTTAGCCGCTTCACTAGTGACTTAGATAA
TATTTCCAATACACTAAACCAAGCATTGATCCAAGTACTATCCAACGTCGCGCTAATGAT
TGGTGTTATCATCATGATGTTCCAACAAAACGTGGAACTAGCCTTCGTTACTCTAATATCT
GCTCCATTTGCAATTATTATTGCGACAGTGATTATTCGAAAAGCACGTAAATTCGTTGAT
GTTCAACAAGATGAACTAGGCGTACTTAACGGCTACATTGACGAAAAAATCTCTGGACA
AAAAATCATTATCACAAATGGTTTAGAAGAAGAAACAATTGACGGCTTTGTTAAACAAA
ACAATATCGTTAAAAACGCCACTTATAAAGGGCAAGTTTACTCCGGTTTACTTTTCCCAA
TGATGCAAGGTATTTCCCTATTAAATACAGCTATCGTTATCTTCTTCGGTGGATGGTTA

abcZ -6: (Strain DS_B2L)

AAATCGACGAACAGAATGCGTATAGGGCTTTTCCGCAAGATGGAAAAACTATCGATTCG
TTTCTTCGATAGCCGCAATGATGGCGAAATGCTTAGTCGTTTTACTAGTGACTTAGATAAT
ATTTCCAATACACTAAACCAAGCATTGATCCAGGTGCTATCCAACGTCGCGCTAATGATT
GGTGTTATCATCATGATGTTCCAACAAAACGTGGAACTAGCCTTCGTTACTTTAATATCT
GCTCCATTTGCGATTATTATTGCAACAGTGATTATTCGAAAAGCACGTAAATTCGTTGAT
GTTCAACAAGATGAACTAGGCGTACTTAACGGCTACATTGACGAAAAAATCTCTGGACA
AAAAATCATTATCACAAATGGTTTAGAAGAAGAAACAATTGACGGCTTTGTTAAACAAA
ACAATATCGTTAAAAACGCCACTTATAAAGGGCAAGTTTACTCCGGTTTACTTTTCCCAA
TGATGCAAGGTATTTCACTATTAAATACAGCTATCGTTATCTTCTTCGGTGGATGGTTA

bglA-4: (Strains DS_14, 25, 31, 53, 63, 68, 81, 82, 84, 85, 88 and PRD5)

AACCAATTCGAAGGCGCTTACAACGTCGATGGAAAAGGACTTTCCGTTCAAGATGTTACT
CCAAAAGGCGGATTTGGTCACATTACTGACGGTCCAACACCAGATAACTTAAAATTAGA
AGGAATTGACTTCTATCATCGCTACAAAGATGACGTGAAACTTTTTGCCGAAATGGGCTT

CAAGGTTTTCCGTACTTCCATCGCTTGGTCCCGTATCTTCCCAAATGGTGACGAAACTGA
GCCAAACGAAGCAGGGCTACAATTTTACGATGATTTATTCGACGAACTTCTAGCACATAA
TATCGAACCACTGATTACTTTATCTCACTATGAAACACCACTTCATTTATCGAAAACTTAC
GACGGATGGGTAAATAGAAAAATGATCGATTTCTATGAA

bglA-10: (Strain DS_80)

AACCAATTCGAAGGCGCTTACAACGTCGATGGAAAAGGACTTTCCGTTCAAGATGTTACT
CCAAAAGGCGGATTCGGTCACATTACTGACGGTCCAACACCAGATAACTTAAAATTAGA
AGGAATCGACTTCTACCATCGCTACAAAGATGACGTGAAACTTTTTGCCGAAATGGGCTT
CAAGGTTTTCCGTACTTCCATCGCTTGGTCCCGTATCTTCCCAAATGGTGACGAAACTGA
GCCAAACGAAGCAGGACTTCAATTTTACGATGATTTATTCGATGAACTTCTAGCACATAA
TATCGAACCACTGATTACTTTATCTCACTATGAAACACCACTTCACTTATCGAAAACTTAC
GACGGCTGGGTAAATAGAAAAATGATCGACTTCTATGAA

bglA-5: (Strain DS_B2L)

AACCAATTCGAAGGCGCTTACAACGTCGATGGAAAAGGACTTTCCGTTCAAGATGTTACT
CCAAAAGGCGGATTCGGTCACATTACTGACGGTCCAACACCAGATAACTTAAAATTAGA
AGGAATCGACTTCTACCATCGCTACAAAGATGACGTGAAACTTTTTGCCGAAATGGGCTT
CAAGGTTTTCCGTACTTCCATCGCTTGGTCCCGTATCTTCCCAAATGGTGACGAAACAGA
GCCAAACGAAGCAGGACTACAATTTTACGATGATTTATTCGATGAACTTCTAGCACATAA
TATCGAACCACTGATTACTTTATCTCACTATGAAACACCACTTCACTTATCGAAAACTTAC
GACGGATGGGTAAATAGAAAAATGATCGACTTCTATGAA

cat-6: (Strains DS_14, 31, 53, 63, 68, 88, PRD5 and B2L)

GCTCGTGGTGCTGGTGCGCACGGGAAATTTGTTACTAAAAAAAGCATGAAAAAATATAC
AATGGCTAAATTTTTGCAAGAAGAAGGAACGGAAACAGAGGTTTTTGCTCGTTTTTCAAC
AGTAATTCATGGGCAACATTCTCCAGAAACATTACGTGATCCACGCGGTTTCTCCGTTAA
GTTTTATACGGAAGAGGGAAATTATGACTTTGTCGGAAATAATTTGCCAGTATTTTTCATT
CGTGATGCGATTAAGTTTCCAGATGTTATTCATTCCTTGAAGCCTGACCCGCGCACAAAT
ATTCAAGATGGCAATCGTTACTGGGATTTCTTTAGCCTTACACCGGAAGCTACGACAATG
ATTATGTACTTATTCAGTGATGAAGGAACGCCGGCTTCTTACCGCGAAGTCCGGGGCTCT
AGTGTTCATGCGTTCAAATGGATTAACGAAGAAGGCAAAACAGTTTATGTAAAACTGCG
CTGGGTT

cat-4: (Strains DS_25, 81, 82, 84 and 85)

GCTCGTGGTGCTGGTGCGCACGGGAAATTTGTCACTAAGAAAAGCATGAAAAAATATAC
AATGGCTAAATTTTTGCAAGAAGAAGGAACGGAAACGGAGGTTTTTGCTCGTTTTTCAAC
AGTAATTCATGGGCAACATTCTCCAGAAACATTACGTGATCCACGAGGTTTCTCCGTTAA
GTTTTATACAGAAGAAGGGAATTATGATTTTGTCGGAAATAATTTGCCGGTATTCTTCATT
CGTGATGCGATTAAGTTTCCGGATGTTATTCATTCCTTGAAGCCTGATCCACGCACAAAT
ATTCAAGATGGCAACCGTTACTGGGATTTCTTTAGCCTTACACCGGAAGCTACGACGATG
ATTATGTACTTATTCAGTGATGAAGGAACGCCGGCTTCTTACCGGGAAATACGTGGCTCT
AGTGTTCATGCGTTCAAATGGATTAACGAAGAAGGCAAAACAGTTTATGTAAAACTGCG
CTGGATT

cat-16: (Strain DS_80)

GCTCGTGGTGCTGGTGCGCACGGGAAATTTGTTACTAAAAAAAGCATGAAAAAATATAC
AATGGCTAAATTTTTGCAAGAAGAAGGAACGGAAACAGAGGTTTTTGCTCGTTTTTCAAC
AGTAATTCATGGGCAACATTCTCCAGAAACATTACGTGATCCACGCGGTTTCTCCGTTAA
GTTTTATACGGAAGAGGGAAATTATGACTTTGTCGGAAATAATTTGCCAGTATTTTTCATT
CGTGATGCGATTAAGTTTCCAGATGTTATTCATTCCTTGAAGCCTGACCCGCGCACAAAT
ATTCAAGATGGCAATCGTTACTGGGATTTCTTTAGCCTTACACCGGAAGCTACGACGATG
ATTATGTACTTATTCAGTGATGAAGGAACGCCGGCTTCTTACCGCGAAGTCCGGGGCTCT

AGTGTTCATGCGTTCAAATGGATTAACGAAGAAGGCAAAACAGTTTATGTAAAATTGCG
CTGGGTT

dapE-4: (Strains DS_14, 31, 53, 63, 88, PRD5 and B2L)

TTGCAAAAGTTGTTAGCTGAACACGGTATTGAGTCCGAAAAGGTACAATACGACGTAGA
CAGAGCCAGCCTAGTAAGCGAAATTGGTTCCAGTAACGAGAAGGTTTTGGCATTTTCAGG
GCATATGGATGTAGTTGATGCGGGTGATGTATCTAAGTGGAAGTTCCCACCTTTTGAAGC
GACAGAGCATGAAGGGAAACTATACGGACGCGGCGCAACGGATATGAAGTCAGGTCTA
GCGGCGATGGTTATTGCAATGATTGAACTTCATGAAGAAAAACAAAAACTAAACGGCAA
GATCAGATTATTAGCAACAGTTGGGGAAGAAATCGGTGAACTTGGAGCAGAACAACTAA
CACAAAAAGGTTACGCAGATGATTTAGATGGTTTAATCATCGGCGAACCGAGCGGACAC
AGAATCGTTTATGCGCATAAAGGTTCCATTAATTATACCGTTAAATCC

dapE-3: (Strains DS_25, 68, 81, 82, 84 and 85)

TTGCAAAAGTTGTTAGCTGAACACGGTATTGAATCCGAAAAGGTACAATACGACGTAGA
CAGAGCCAGCCTAGTTAGCGAAATTGGTTCCAGTGACGAGAAAGTTTTGGCGTTTTCAGG
GCATATGGATGTCGTTGATGCGGGTGATGTCTCGAAGTGGAAGTTCCCACCTTTTGAAGC
AGCAGAGCATGAAGGGAAAATATACGGACGTGGCGCGACGGATATGAAGTCAGGTCTA
GCGGCGATGATTATTGCAATGATTGAGCTTCATGAAGAAAAACAAAAACTAAATGGCAA
AATTAGATTATTAGCAACGGTTGGTGAAGAAGTCGGTGAACTTGGAGCCGAACAACTAA
CGCAAAAAGGTTACGCAGATGATTTAGATGGCTTGATTATCGGCGAACCGAGTGGACAC
CGGATTGTTTATGCGCATAAAGGTTCCATTAATTATACCGTTAAATCC

dapE-7: (Strain DS_80)

TTGCAAAAGTTGTTAGCTGAACACGGTATTGAGTCCGAAAAGGTACAATACGACGTAGA
CAGAGCTAGCCTAGTAAGCGAAATTGGTTCCAGTAACGAGAAGGTTTTGGCATTTTCAGG
GCATATGGATGTAGTTGATGCGGGTGATGTATCTAAGTGGAAGTTCCCACCTTTTGAAGC
GACAGAGCATGAAGGGAAACTATACGGACGCGGCGCAACGGATATGAAGTCAGGTCTA
GCGGCGATGGTTATTGCAATGATTGAACTTCATGAAGAAAAACAAAAACTAAACGGCAA
GATCAGATTATTAGCAACAGTTGGGGAAGAAATCGGTGAACTTGGAGCAGAACAACTAA
CACAAAAAGGTTACGCAGATGATTTAGATGGTTTAATCATCGGTGAACCGAGCGGACAC
AGAATCGTTTATGCGCATAAAGGTTCCATTAATTATACCGTTAAATCC

dat-5: (Strains DS_14, 31, 53, 63, 80, 88 and PRD5)

GAAGTAGTTCGTCTATATAATGGAAAATTCTTTACTTATAATGAACACATTGATCGCTTA
TATGCTAGTGCAGCAAAAATTGACTTAGTTATTCCTTATTCCAAAGAAGAGCTACGTGAA
TTACTTGAAAAATTAGTTGCCGAAAATAATATCAATACAGGGAATGTCTATTTACAAGTG
ACTCGTGGTGTTCAAAACCCACGTAATCATGTAATCCCTGATGATTTCCCTCTAGAAGGC
GTTTTAACAGCAGCAGCTCGTGAAGTACCTAGAAACGAGCGTCAATTCGTTGAAGGTGG
AACGGCTATTACAGAAGAAGATGTGCGCTGGTTACGCTGTGATATTAAGAGCTTAAACCT
TTTAGGAAATATTCTAGCAAAAAATAAAGCACATCAACAAAATGCTTTGGAAGCTATTTT
ACATCGCGGGGAACAAGTAACGGAATGTTCTGCTTCAAACGTTTCTATTATT

dat-2: (Strains DS_25, 68, 81, 82, 84 and 85)

GAAGTAGTTCGTCTATATAATGGAAAATTCTTCACTTATAATGAACACATTGATCGTTTA
TATGCGAGTGCAGCAAAAATTGACTTAGTTATTCCTTATTCGAAAGAAGAGTTACGAGCG
TTACTTGAAAAATTAGTTGCTGAAAATAATATTAATACAGGAAATGTCTATTTACAAGTG
ACTCGAGGTGTTCAAAACCCGCGTAATCACGTTATGCCAGATGATTTCCCGCTGGAAGGC
GTTTTAACAGCAGCAGCTCGTGAAGTACCAAGAAATGAACAACAATTTGTGCAAGGTGG
ACCAGTAATTACAGAAGAAGATGTTCGTTGGTTACGCTGTGACATCAAGAGTTTGAATTT
ACTTGGAAACATTTTAGCAAAAAACAAAGCACATCAACAAAATGCGTTAGAAGCTGTTT
TACACCGCGGAGAGCAAGTAACTGAGTGTTCAGCTTCCAATATTTCTATTATT

***dat*-1: (Strain DS_B2L)**

GAAGTAGTTCGTCTATATAATGGAAAATTCTTTACTTATAATGAACACATTGATCGCTTA
TATGCTAGTGCAGCAAAAATTGACTTAGTTATTCCTTATTCCAAAGAAGAGCTACGTGAA
TTACTTGAAAAATTAGTTGCCGAAAATAATATCAATACAGGGAATGTCTATTTACAAGTG
ACTCGTGGTGTTCAAAACCCACGTAATCATGTAATCCCTGATGATTTCCCTCTAGAAGGC
GTTTTAACAGCAGCAGCTCGTGAAGTACCTAGAAACGAGCGTCAATTCGTTGAAGGTGG
AACGGCGATTACAGAAGAAGATGTGCGCTGGTTACGCTGTGATATTAAGAGCTTAAACC
TTTTAGGAAATATTCTAGCAAAAAATAAAGCACATCAACAAAATGCTTTGGAAGCTATTT
TACATCGCGGGGAACAAGTAACGGAATGTTCTGCTTCAAACGTTTCTATTATT

***ldh*-1: (Strains DS_14, 25, 31, 53, 63, 81, 82, 84, 85, 88 and PRD5)**

TATAGCGACTGCCACGATGCGGACTTAGTTGTTGTAACTGCCGGGACTGCACAAAAACCT
GGTGAAACTCGTTTAGATTTAGTAAATCGTAATATTAAAATCATGAAAGGCATCGTGGAT
GAAGTAATGGCTAGCGGATTTGACGGTATCTTCTTAATCGCTTCTAACCCAGTAGATATC
TTAACTTACGCTACATGGAAATTCTCAGGTCTTCCAAAAGAACGTGTTATCGGTTCTGGA
ACAAGCCTTGATACAGCACGTTTCCGTATGTCAATTGCCGACTATCTAAAAGTAGATGCT
CGTAACGTCCATGGTTACATCCTTGGCGAACACGGCGATACAGAATTCCCAGCATGGAGC
CACACAACTGTCGGCGGTCTTCCAATCACTGAATGGATTAGCGAAGATGAACAAGGTGC
AATGGATACTATTTTCGTAAGTGTTCGTGATGCA

***ldh*-2: (Strains DS_68 and 80)**

TATAGCGACTGCCACGATGCGGACCTAGTTGTTGTAACTGCCGGTACTGCTCAAAAACCT
GGTGAAACTCGTTTAGATCTAGTAAATCGTAATATCAAAATCATGAAAGGTATCGTGGAT
GAAGTTATGGCAAGCGGATTTGATGGTATCTTCTTAATCGCTTCTAACCCAGTAGACATC
TTAACTTACGCTACATGGAAATTCTCAGGTCTTCCAAAAGAACGTGTTATCGGTTCTGGA
ACAAGCCTTGATACAGCACGTTTCCGTATGTCAATTGCTGACTATCTAAAAGTAGATGCT
CGTAACGTCCATGGTTACATCCTTGGCGAACACGGCGATACAGAATTCCCAGCATGGAGC
CACACAACTGTCGGCGGCCTTCCAATTACTGAATGGATTAGCGAAGATGAACAAGGTGC
AATGGATACTATTTTCGTAAGTGTTCGTGATGCA

***ldh*-4: (Strain DS_B2L)**

TATAGCGACTGCCACGATGCGGACTTAGTTGTTGTAACTGCCGGTACTGCTCAAAAACCT
GGTGAAACTCGTTTAGATCTAGTAAATCGTAATATCAAAATCATGAAAGGCATCGTGGAT
GAAGTTATGGCAAGCGGATTTGATGGTATCTTCTTAATCGCTTCTAACCCAGTAGACATC
TTAACTTACGCTACATGGAAATTCTCAGGTCTTCCAAAAGAACGTGTTATCGGTTCTGGA
ACAAGCCTTGATACAGCACGTTTCCGTATGTCAATTGCTGACTATCTAAAAGTAGATGCT
CGTAACGTCCATGGTTACATCCTTGGCGAACACGGCGATACAGAATTCCCAGCATGGAGC
CACACAACTGTCGGCGGCCTTCCAATTACTGAATGGATTAGCGAAGATGAACAAGGTGC
AATGGATACTATTTTCGTAAGTGTTCGTGATGCA

***lhkA*-1: (Strains DS_14, 31, 53, 63, 80, 88, PRD5 and B2L)**

TATCCAACACAGATGAATCAGCCTTTACCTAAGGATTTCTCTATTTCTGCGGATGATAAG
AAAAAGCTTGAAAGTGGTGAAACGGTTAGTAAGAAAATAGATAATCGCTTTAACAAAGA
AATGACAATTGTGTACGTCCCAATAATGAATGGCGATAAATTTGTCGGTTCTATCGTGCT
GAATTCACCCATTAGCGGTACGGAGCAAGTAATTGGCACGATTAACCGCTATATGTTCTA
CACTATTTTACTTTCTATAACGGTAGCACTTATTCTTAGCGCAATCTTGTCCAAACTACAA
GTAAATCGAATCAACAAACTACGAGCAGCGACAAAAGACGTTATTCAAGGCAATTACAA
CGCTCGCTTGAAGGAAAATAATTTTGATGAAATTGGTGCACTCGCCATTGATTTCAATAA
AATGACACAAACCCTTGAAACATCTCAAGAAGAAATTGAACGACAAGAGAAACGGAGA
CGC

***lhkA*-5: (Strains DS_25, 68, 81, 82, 84 and 85)**

TATCCAACACAGATGAATCAGCCGTTACCAAAGGATTTCTCTATTTCTTCGGATGATAAG
AAAAAGCTTGAAAGTGGCGAAACAGTTAGTAAGAAAATAGATAATCGCTTTAATAAAGA
AATGACAATTGTGTACGTCCCAATAATGAATGGCGACAAATTTGTCGGTTCTATCGTGCT
CAATTCACCTATTAGCGGTACGGAGCAAGTAATTGGTACGATTAACCGCTATATGTTCTA
CACTATTTTACTTTCTATAACGGTAGCACTTATTCTTAGCGCAATCTTGTCCAAACTACAA
GTAAATCGAATCAACAAACTACGAGCAGCGACAAAAGACGTTATTCAAGGCAATTACAA
AGCTCGATTGAAGGAAAATAATTTTGATGAAATTGGTGCACTTGCCATTGATTTCAATAA
AATGACACAAACCCTTGAAACATCTCAAGAAGAAATAGAACGACAAGAGAAGCGGAGA
CGC

APPENDIX 4

BIOFILM MEASUREMENT DATA

A4.1 Biofilm measurements of *Listeria monocytogenes* strains cultured at 10, 20, 25 and 37°C after 24 hours (120 h for the 10°C treatment) incubation obtained using a colourimetric microtitre plate assay. Measurement values (absorbance at 595 nm) are presented as the mean and standard deviation of three replicates and are arranged in ascending order. The top 10% biofilm production for each condition is shaded red. The top 20% are shaded grey. The bottom 10% biofilm production for each condition is shaded gold. The bottom 20% is shaded green.

10 Degree; 120h			20 Degree; 24h			25 Degree; 24h			37 Degree; 24h		
Strain	Mean	StDev	Strain	Mean	StDev	Strain	Mean	StDev	Strain	Mean	StDev
FW06-20	0.009	0.003	83-2795	0.007	0.005	102-695-S1-154	0.000	0.000	FW06-40	0.024	0.009
FW06-46	0.012	0.009	FW06-20	0.009	0.003	Joyce	0.000	0.000	FW06-14	0.043	0.002
69-1793	0.015	0.017	69-577	0.019	0.008	FW06-34	0.004	0.001	L1	0.043	0.043
FW06-26	0.015	0.009	Joyce	0.020	0.003	FW06-14	0.004	0.001	FW06-9	0.049	0.004
102-265-S3-745	0.019	0.010	L1	0.021	0.007	FW04-25	0.004	0.002	FW06-44	0.059	0.033
FW06-28	0.020	0.009	102-231-S7-232	0.021	0.004	FW06-26	0.008	0.008	FW06-34	0.070	0.045
FW03-35	0.021	0.009	FW06-17	0.022	0.024	FW03-35	0.009	0.002	FW06-7	0.071	0.021
69-577	0.022	0.033	102-695-S1-154	0.022	0.005	114-997-S7-63	0.012	0.006	FW06-46	0.077	0.028
102-195-S1-242	0.023	0.010	102-231-S7-566	0.023	0.002	102-231-S7-566	0.015	0.006	FW06-3	0.079	0.021
FW06-7	0.023	0.022	FW04-25	0.023	0.005	FW06-40	0.015	0.003	FW06-38	0.084	0.014
FW06-40	0.024	0.013	FW03-35	0.024	0.003	FW06-9	0.015	0.005	102-695-S1-154	0.086	0.037
102-231-S7-566	0.026	0.012	87-1599	0.025	0.001	102-265-S3-745	0.017	0.008	102-231-S7-232	0.087	0.016

70-1700	0.032	0.027	62-2853	0.027	0.036	102-241-S1-349	0.018	0.004	FW06-43	0.091	0.027
Joyce	0.034	0.031	FW04-19	0.031	0.005	FW06-46	0.019	0.004	114-997-S7-63	0.092	0.020
102-695-S1-154	0.034	0.018	114-997-S7-63	0.034	0.004	87-1599	0.020	0.002	102-265-S3-352	0.097	0.004
FW06-12	0.035	0.008	ATCC 19115	0.036	0.003	102-231-S7-232	0.022	0.009	FW06-15	0.099	0.043
102-231-S7-232	0.037	0.006	70-0421	0.036	0.020	FW04-19	0.023	0.013	FW04-19	0.106	0.014
FW06-39	0.039	0.039	L2	0.037	0.007	Liver	0.026	0.007	FW03-32	0.106	0.015
FW06-29	0.039	0.011	FW03-32	0.039	0.015	FW06-44	0.027	0.012	Joyce	0.109	0.008
102-241-S1-349	0.042	0.008	FW04-17	0.042	0.009	LO28	0.029	0.009	FW04-25	0.109	0.036
FW06-34	0.043	0.012	102-265-S3-352	0.047	0.032	102-195-S1-242	0.029	0.012	FW06-36	0.110	0.041
FW06-42	0.043	0.010	LO28	0.051	0.005	69-577	0.033	0.018	FW06-24	0.122	0.021
ATCC 19115	0.044	0.018	ATCC 19114	0.051	0.012	102-265-S3-352	0.033	0.009	102-241-S1-349	0.132	0.014
FW03-32	0.046	0.018	102-195-S1-242	0.053	0.014	FW06-16	0.036	0.002	FW04-20	0.135	0.013
FW06-27	0.049	0.010	102-241-S1-349	0.053	0.004	FW04-20	0.036	0.006	FW04-21	0.136	0.018
FW06-18	0.050	0.017	FW06-34	0.054	0.023	FW06-43	0.038	0.014	87-1599	0.160	0.012
FW06-47	0.050	0.020	69-1793	0.058	0.037	L2	0.040	0.010	LO28	0.163	0.026
FW06-14	0.051	0.043	FW06-40	0.070	0.001	ATCC 19114	0.041	0.026	102-231-S7-566	0.164	0.028
FW06-21	0.051	0.033	Liver	0.072	0.013	FW06-41	0.041	0.005	FW06-16	0.168	0.014
FW06-10	0.052	0.042	FW06-44	0.074	0.014	FW03-32	0.041	0.001	ATCC 19114	0.169	0.050
62-2853	0.052	0.013	64-1495	0.076	0.035	FW06-18	0.042	0.015	FW06-26	0.175	0.014
FW06-13	0.053	0.013	102-265-S3-745	0.079	0.025	ATCC 19115	0.042	0.014	FW03-35	0.175	0.031
FW04-25	0.056	0.026	ScottA	0.081	0.016	FW06-6	0.046	0.014	ScottA	0.183	0.028
FW06-35	0.057	0.038	70-1700	0.086	0.026	69-1793	0.048	0.029	102-265-S3-745	0.185	0.021
FW04-19	0.057	0.030	FW06-10	0.092	0.024	ScottA	0.053	0.012	FW06-11	0.185	0.073
FW06-44	0.057	0.003	FW06-12	0.093	0.002	FW04-21	0.054	0.003	DS_84	0.188	0.029
FW06-17	0.058	0.021	FW06-46	0.093	0.011	L1	0.056	0.033	DS_31	0.191	0.034
LO28	0.059	0.007	FW06-26	0.099	0.029	FW06-12	0.057	0.011	69-577	0.193	0.119

FW04-20	0.061	0.009	FW06-14	0.105	0.007	FW06-15	0.057	0.015	DS_53	0.198	0.009
FW06-11	0.062	0.027	FW06-23	0.107	0.033	FW06-33	0.063	0.017	PRD5	0.198	0.016
FW06-32	0.065	0.038	FW06-33	0.111	0.014	70-1700	0.063	0.032	L2	0.199	0.005
FW06-6	0.066	0.004	FW06-35	0.113	0.022	FW04-17	0.064	0.002	DS_80	0.199	0.015
FW06-9	0.067	0.033	DS_B2L	0.114	0.020	FW06-7	0.065	0.004	DS_25	0.201	0.025
87-1599	0.067	0.039	FW06-7	0.115	0.008	FW06-24	0.068	0.013	FW06-20	0.207	0.075
FW06-31	0.071	0.050	FW06-21	0.117	0.005	FW06-38	0.075	0.050	102-195-S1-242	0.207	0.020
FW06-5	0.072	0.044	FW06-5	0.119	0.018	FW06-21	0.076	0.011	FW06-2	0.208	0.061
FW06-16	0.073	0.055	FW06-47	0.125	0.009	DS_84	0.083	0.017	DS_88	0.209	0.003
83-2795	0.077	0.037	FW06-42	0.127	0.008	FW06-10	0.085	0.018	DS_85	0.210	0.021
FW04-21	0.082	0.010	FW06-15	0.128	0.030	DS_25	0.085	0.011	B2L	0.213	0.023
FW06-25	0.085	0.017	DS_53	0.130	0.024	FW06-11	0.087	0.024	FW06-25	0.215	0.037
FW06-43	0.088	0.029	FW06-28	0.131	0.010	FW06-32	0.091	0.030	DS_68	0.217	0.047
L2	0.089	0.021	FW06-18	0.135	0.036	FW06-35	0.091	0.027	69-1793	0.225	0.031
FW06-41	0.092	0.005	DS_85	0.135	0.037	FW06-29	0.098	0.029	FW06-35	0.229	0.041
FW06-33	0.101	0.037	FW06-11	0.137	0.032	DS_88	0.102	0.021	ATCC 19115	0.237	0.049
FW06-37	0.102	0.008	FW06-41	0.138	0.049	FW06-28	0.103	0.007	DS_81	0.237	0.072
FW06-3	0.104	0.006	FW06-27	0.139	0.018	DS_63	0.107	0.018	DS_63	0.252	0.114
FW06-45	0.106	0.038	FW04-21	0.140	0.013	DS_80	0.111	0.011	62-2853	0.263	0.035
FW06-30	0.107	0.011	DS_80	0.141	0.026	DS_31	0.111	0.018	Liver	0.268	0.016
FW06-50	0.107	0.013	FW06-24	0.142	0.004	FW06-5	0.112	0.039	FW06-29	0.274	0.061
FW06-24	0.108	0.013	FW04-20	0.142	0.029	83-2795	0.112	0.012	FW06-27	0.289	0.038
ATCC 19114	0.109	0.015	DS_88	0.142	0.030	PRD5	0.115	0.013	DS_82	0.295	0.152
FW06-38	0.113	0.020	FW06-16	0.142	0.016	DS_B2L	0.116	0.019	DS_14	0.303	0.016
FW06-36	0.119	0.027	FW06-43	0.148	0.028	DS_85	0.122	0.008	FW04-17	0.304	0.013
FW06-2	0.119	0.039	DS_84	0.152	0.023	DS_14	0.123	0.057	FW06-30	0.312	0.011

FW06-49	0.120	0.037	FW06-48	0.155	0.012	64-1495	0.125	0.024	83-2795	0.317	0.047
FW06-15	0.124	0.046	FW06-6	0.156	0.034	FW06-17	0.129	0.017	FW06-42	0.318	0.030
FW06-48	0.125	0.056	DS_14	0.157	0.042	FW06-42	0.130	0.033	FW06-17	0.331	0.072
FW04-17	0.126	0.019	DS_31	0.160	0.024	DS_53	0.132	0.010	FW06-5	0.336	0.029
L1	0.130	0.021	FW06-32	0.164	0.044	DS_81	0.133	0.048	FW06-28	0.346	0.058
FW06-19	0.133	0.033	FW06-38	0.164	0.015	FW06-27	0.138	0.061	FW06-19	0.348	0.035
ScottA	0.133	0.039	FW06-3	0.168	0.031	DS_68	0.146	0.041	FW06-32	0.353	0.027
FW06-8	0.145	0.050	FW06-29	0.174	0.018	DS_82	0.161	0.036	FW06-23	0.356	0.038
102-265-S3-352	0.149	0.033	FW06-30	0.184	0.030	FW06-20	0.177	0.052	FW06-21	0.373	0.087
114-997-S7-63	0.153	0.034	DS_68	0.187	0.021	62-2853	0.184	0.027	FW06-45	0.377	0.017
FW06-23	0.158	0.064	DS_63	0.194	0.049	FW06-45	0.203	0.011	FW06-50	0.390	0.025
FW06-22	0.163	0.030	FW06-36	0.195	0.010	FW06-30	0.207	0.034	64-1495	0.400	0.043
FW06-4	0.187	0.043	DS_81	0.199	0.015	FW06-49	0.217	0.039	FW06-1	0.404	0.074
70-0421	0.199	0.049	FW06-1	0.203	0.032	FW06-22	0.226	0.009	70-1700	0.417	0.060
DS_80	0.199	0.009	DS_25	0.206	0.015	FW06-19	0.235	0.095	FW06-33	0.452	0.062
DS_81	0.199	0.007	FW06-9	0.208	0.166	FW06-2	0.249	0.087	FW06-39	0.465	0.023
DS_25	0.203	0.009	DS_PRD5	0.209	0.042	FW06-48	0.272	0.015	FW06-47	0.467	0.087
DS_53	0.207	0.026	FW06-49	0.211	0.015	FW06-47	0.295	0.060	FW06-22	0.476	0.099
DS_PRD5	0.208	0.010	DS_82	0.236	0.030	FW06-23	0.312	0.097	FW06-10	0.495	0.206
DS_68	0.209	0.015	FW06-13	0.237	0.009	FW06-13	0.321	0.021	FW06-37	0.511	0.028
DS_84	0.209	0.019	FW06-50	0.254	0.006	FW06-1	0.327	0.069	FW06-13	0.513	0.068
DS_82	0.210	0.008	FW06-19	0.257	0.027	FW06-39	0.327	0.023	70-0421	0.534	0.056
DS_B2L	0.218	0.032	FW06-39	0.257	0.011	FW06-36	0.336	0.015	FW06-48	0.606	0.035
DS_85	0.218	0.023	FW06-2	0.304	0.191	FW06-25	0.339	0.050	FW06-49	0.636	0.234
DS_88	0.220	0.019	FW06-45	0.307	0.015	FW06-31	0.367	0.016	FW06-6	0.831	0.161
Liver	0.222	0.045	FW06-22	0.314	0.084	70-0421	0.380	0.158	FW06-31	0.834	0.196

DS_63	0.228	0.027	FW06-8	0.384	0.057	FW06-50	0.415	0.018	FW06-4	0.934	0.080
DS_31	0.236	0.036	FW06-4	0.387	0.015	FW06-4	0.513	0.072	FW06-8	1.114	0.061
DS_14	0.242	0.021	FW06-25	0.409	0.169	FW06-37	0.552	0.033	FW06-12	1.315	0.375
64-1495	0.250	0.087	FW06-31	0.437	0.081	FW06-3	0.635	0.339	FW06-18	1.509	0.257
FW06-1	0.336	0.094	FW06-37	0.482	0.010	FW06-8	0.792	0.185	FW06-41	1.958	0.719

A4.2 Biofilm measurements of *Listeria monocytogenes* strains cultured at 10, 20, 25 and 37°C after 48 hours (144 h for the 10°C treatment) incubation obtained using a colourimetric microtitre plate assay. Measurement values (absorbance at 595 nm) are presented as the mean and standard deviation of three replicates and are arranged in ascending order. The top 10% biofilm production for each condition is shaded red. The top 20% are shaded grey. The bottom 10% biofilm production for each condition is shaded gold. The bottom 20% is shaded green.

10 Degree; 144h			20 Degree; 48h			25 Degree; 48h			37 Degree; 48h		
Strain	Mean	StDev	Strain	Mean	StDev	Strain	Mean	StDev	Strain	Mean	StDev
102-695-S1-154	0.004	0.002	102-695-S1-154	0.001	0.001	69-577	0.019	0.012	L1	0.043	0.019
Joyce	0.004	0.004	102-231-S7-566	0.015	0.002	102-695-S1-154	0.020	0.003	102-695-S1-154	0.073	0.011
FW04-25	0.005	0.003	102-231-S7-232	0.018	0.008	FW03-35	0.023	0.001	LO28	0.078	0.015
FW06-20	0.006	0.004	69-577	0.019	0.025	Joyce	0.024	0.007	FW03-35	0.081	0.027
102-231-S7-566	0.009	0.008	FW03-35	0.019	0.007	102-231-S7-232	0.030	0.007	FW06-14	0.085	0.001
102-265-S3-745	0.011	0.008	FW04-25	0.020	0.002	69-1793	0.034	0.017	102-231-S7-566	0.092	0.046
87-1599	0.012	0.004	87-1599	0.022	0.010	FW04-20	0.037	0.005	114-997-S7-63	0.095	0.052
102-195-S1-242	0.014	0.002	Joyce	0.022	0.009	114-997-S7-63	0.039	0.015	FW04-25	0.101	0.016
69-577	0.018	0.019	L2	0.023	0.005	102-265-S3-745	0.040	0.021	Joyce	0.103	0.007
FW03-35	0.018	0.020	FW06-20	0.029	0.009	102-241-S1-349	0.045	0.028	FW06-20	0.105	0.015
L1	0.019	0.015	L1	0.030	0.014	FW04-19	0.048	0.015	FW03-32	0.112	0.013
FW06-46	0.020	0.013	FW03-32	0.031	0.006	102-231-S7-566	0.049	0.027	FW04-19	0.116	0.015
ATCC 19115	0.021	0.010	ATCC 19115	0.034	0.008	70-1700	0.050	0.021	87-1599	0.119	0.043
FW03-32	0.022	0.009	114-997-S7-63	0.035	0.014	102-265-S3-352	0.057	0.010	FW04-20	0.121	0.024
FW04-19	0.023	0.006	102-265-S3-352	0.040	0.019	FW04-25	0.060	0.017	102-241-S1-349	0.131	0.004
FW06-14	0.025	0.015	FW04-19	0.042	0.005	L1	0.061	0.005	69-1793	0.146	0.078
FW04-20	0.026	0.007	LO28	0.043	0.012	83-2795	0.071	0.031	Liver	0.148	0.019

102-241-S1-349	0.026	0.009	102-241-S1-349	0.044	0.011	ATCC 19114	0.074	0.007	102-265-S3-352	0.164	0.083
FW06-33	0.028	0.011	102-195-S1-242	0.044	0.010	DS_68	0.074	0.026	102-231-S7-232	0.169	0.032
FW06-5	0.029	0.011	62-2853	0.054	0.002	102-195-S1-242	0.078	0.027	FW06-44	0.174	0.017
FW06-40	0.030	0.032	70-0421	0.057	0.036	FW06-17	0.078	0.037	62-2853	0.179	0.039
FW06-34	0.031	0.044	70-1700	0.061	0.034	L2	0.078	0.023	102-265-S3-745	0.183	0.030
FW06-26	0.035	0.033	83-2795	0.070	0.004	FW03-32	0.083	0.012	102-195-S1-242	0.189	0.065
FW06-42	0.035	0.013	102-265-S3-745	0.071	0.017	ATCC 19115	0.085	0.024	ATCC 19114	0.194	0.047
FW06-43	0.038	0.015	ScottA	0.072	0.007	LO28	0.087	0.021	FW06-27	0.197	0.012
70-1700	0.039	0.044	FW06-17	0.079	0.012	FW06-34	0.088	0.002	L2	0.198	0.025
FW06-28	0.039	0.011	ATCC 19114	0.093	0.042	Liver	0.094	0.034	DS_63	0.200	0.059
FW06-12	0.042	0.012	64-1495	0.093	0.035	87-1599	0.096	0.031	ScottA	0.220	0.028
62-2853	0.043	0.002	FW04-20	0.099	0.005	FW04-21	0.100	0.005	70-1700	0.237	0.034
FW06-32	0.046	0.011	FW06-46	0.100	0.006	FW06-44	0.101	0.003	FW06-17	0.238	0.104
LO28	0.046	0.005	FW06-14	0.104	0.005	ScottA	0.113	0.032	DS_25	0.242	0.052
FW04-21	0.049	0.004	FW04-21	0.107	0.012	FW06-20	0.118	0.070	FW04-21	0.246	0.037
FW06-10	0.051	0.023	FW04-17	0.112	0.046	FW06-9	0.128	0.002	DS_81	0.267	0.100
FW06-17	0.054	0.029	FW06-44	0.117	0.059	FW04-17	0.136	0.018	DS_88	0.268	0.056
102-231-S7-232	0.055	0.040	FW06-43	0.119	0.022	64-1495	0.137	0.045	FW06-9	0.271	0.018
FW06-27	0.058	0.011	FW06-12	0.123	0.017	62-2853	0.139	0.051	83-2795	0.280	0.084
83-2795	0.059	0.009	FW06-5	0.134	0.007	FW06-14	0.144	0.056	FW06-40	0.282	0.017
ATCC 19114	0.061	0.010	FW06-40	0.134	0.019	DS_PRD5	0.149	0.034	69-577	0.290	0.066
FW06-18	0.061	0.024	FW06-34	0.142	0.028	FW06-27	0.158	0.023	FW06-34	0.290	0.023
69-1793	0.062	0.082	FW06-10	0.144	0.016	DS_25	0.159	0.051	DS_82	0.302	0.094
FW06-7	0.063	0.031	69-1793	0.147	0.021	FW06-26	0.165	0.008	DS_B2L	0.311	0.075
FW06-44	0.063	0.040	FW06-33	0.155	0.042	70-0421	0.168	0.104	ATCC 19115	0.312	0.068
FW06-47	0.063	0.022	FW06-15	0.155	0.080	FW06-46	0.171	0.078	FW04-17	0.312	0.059

FW06-29	0.071	0.018	FW06-19	0.157	0.045	FW06-16	0.186	0.049	FW06-26	0.313	0.028
L2	0.074	0.006	FW06-42	0.157	0.025	DS_88	0.193	0.011	FW06-2	0.315	0.050
FW06-21	0.078	0.033	FW06-18	0.163	0.006	FW06-38	0.196	0.030	DS_31	0.322	0.094
FW04-17	0.082	0.020	FW06-16	0.163	0.070	DS_85	0.198	0.088	FW06-7	0.323	0.026
64-1495	0.092	0.054	FW06-32	0.167	0.022	DS_80	0.215	0.042	FW06-33	0.330	0.114
FW06-48	0.095	0.044	FW06-22	0.171	0.028	FW06-42	0.215	0.011	FW06-29	0.333	0.081
ScottA	0.095	0.056	FW06-26	0.172	0.087	FW06-32	0.217	0.034	FW06-23	0.337	0.055
70-0421	0.100	0.041	FW06-47	0.174	0.007	DS_53	0.223	0.022	FW06-46	0.341	0.014
FW06-16	0.103	0.037	Liver	0.187	0.038	FW06-33	0.225	0.007	FW06-32	0.359	0.024
Liver	0.106	0.016	FW06-21	0.192	0.020	FW06-7	0.228	0.017	DS_84	0.364	0.082
102-265-S3-352	0.110	0.017	FW06-48	0.193	0.009	DS_31	0.231	0.125	64-1495	0.365	0.158
FW06-15	0.121	0.058	FW06-2	0.196	0.016	FW06-12	0.233	0.012	FW06-24	0.378	0.025
FW06-2	0.124	0.020	DS_53	0.203	0.013	FW06-18	0.235	0.033	FW06-19	0.398	0.068
DS_68	0.128	0.023	FW06-29	0.204	0.021	DS_14	0.236	0.047	DS_68	0.401	0.022
FW06-49	0.130	0.069	FW06-7	0.205	0.046	DS_84	0.238	0.055	FW06-49	0.402	0.114
FW06-22	0.130	0.024	DS_80	0.208	0.014	FW06-35	0.241	0.048	FW06-22	0.404	0.105
FW06-19	0.132	0.040	DS_84	0.211	0.020	FW06-11	0.243	0.052	FW06-3	0.413	0.035
FW06-23	0.133	0.054	FW06-6	0.215	0.042	FW06-15	0.247	0.005	FW06-5	0.424	0.036
FW06-6	0.137	0.015	FW06-49	0.216	0.053	DS_82	0.250	0.032	FW06-28	0.429	0.009
114-997-S7-63	0.139	0.020	DS_63	0.219	0.022	FW06-29	0.253	0.016	FW06-25	0.438	0.022
FW06-30	0.142	0.064	DS_B2L	0.220	0.036	DS_B2L	0.253	0.142	FW06-15	0.456	0.030
FW06-41	0.146	0.031	FW06-23	0.222	0.083	FW06-24	0.264	0.048	DS_85	0.457	0.067
FW06-35	0.161	0.032	FW06-28	0.230	0.124	FW06-23	0.277	0.095	FW06-30	0.463	0.013
FW06-38	0.178	0.027	FW06-27	0.246	0.086	FW06-40	0.280	0.132	FW06-16	0.467	0.010
FW06-11	0.178	0.009	FW06-3	0.251	0.111	FW06-28	0.283	0.009	FW06-35	0.475	0.009
DS_63	0.180	0.057	DS_88	0.257	0.003	FW06-1	0.295	0.073	FW06-38	0.477	0.082

FW06-36	0.191	0.030	FW06-11	0.265	0.115	FW06-5	0.309	0.066	FW06-11	0.482	0.022
DS_53	0.193	0.018	FW06-30	0.270	0.013	FW06-43	0.326	0.035	DS_14	0.484	0.043
FW06-13	0.200	0.011	DS_31	0.273	0.033	DS_63	0.359	0.210	DS_80	0.488	0.082
DS_84	0.201	0.011	FW06-35	0.275	0.023	FW06-2	0.374	0.016	DS_PRD5	0.488	0.030
FW06-3	0.202	0.070	DS_81	0.275	0.032	FW06-10	0.393	0.029	FW06-36	0.499	0.073
DS_81	0.202	0.035	DS_68	0.277	0.047	FW06-21	0.393	0.150	FW06-42	0.543	0.141
DS_88	0.207	0.024	FW06-24	0.285	0.084	FW06-22	0.406	0.050	FW06-48	0.547	0.039
FW06-24	0.208	0.087	DS_PRD5	0.307	0.039	FW06-19	0.415	0.051	DS_53	0.576	0.183
DS_82	0.208	0.050	FW06-36	0.322	0.020	FW06-30	0.440	0.024	FW06-37	0.576	0.067
DS_25	0.221	0.038	DS_82	0.323	0.037	FW06-48	0.479	0.025	70-0421	0.579	0.025
DS_31	0.221	0.026	DS_25	0.348	0.061	FW06-47	0.482	0.048	FW06-43	0.596	0.117
B2L	0.227	0.065	FW06-45	0.349	0.053	FW06-3	0.526	0.226	FW06-21	0.611	0.112
FW06-45	0.233	0.129	FW06-38	0.352	0.080	FW06-45	0.535	0.027	FW06-39	0.612	0.015
DS_PRD5	0.234	0.014	FW06-13	0.355	0.037	FW06-31	0.540	0.005	FW06-50	0.621	0.043
DS_85	0.243	0.051	FW06-41	0.367	0.122	DS_81	0.542	0.228	FW06-45	0.634	0.052
DS_80	0.250	0.034	DS_85	0.371	0.051	FW06-13	0.550	0.019	FW06-47	0.681	0.038
FW06-39	0.251	0.028	FW06-1	0.379	0.078	FW06-25	0.552	0.078	FW06-31	0.699	0.024
FW06-50	0.264	0.065	FW06-39	0.384	0.058	FW06-50	0.617	0.012	FW06-1	0.822	0.226
FW06-31	0.308	0.154	DS_14	0.403	0.018	FW06-49	0.632	0.091	FW06-13	0.827	0.045
DS_14	0.311	0.019	FW06-50	0.407	0.023	FW06-36	0.638	0.020	FW06-4	0.870	0.140
FW06-1	0.333	0.062	FW06-25	0.443	0.109	FW06-6	0.758	0.093	FW06-8	0.876	0.034
FW06-25	0.339	0.154	FW06-31	0.449	0.101	FW06-4	0.765	0.016	FW06-6	0.888	0.073
FW06-4	0.363	0.014	FW06-4	0.466	0.005	FW06-39	0.825	0.124	FW06-41	1.266	0.171
FW06-8	0.432	0.279	FW06-8	0.514	0.152	FW06-8	0.954	0.118	FW06-18	1.377	0.037
FW06-9	0.463	0.064	FW06-9	0.634	0.141	FW06-37	0.961	0.216	FW06-12	1.412	0.065
FW06-37	0.474	0.279	FW06-37	0.814	0.256	FW06-41	0.962	0.024	FW06-10	1.735	0.283

APPENDIX 5
MUDPIT DATA

MuDPIT data for Chapters 4 and 5 (Appendix 5.1 – 5.3) are provided on the accompanying CD-ROM.